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BIOMARKERS OF EXPOSURE TO TOXIC SUBSTANCES
Volume 4: Metabonomics
Biomarkers to Liver and Organ Damage

Nicholas DelRaso
Randel Reitcheck
Deirdre Mahle
Biosciences and Protection Division
Applied Biotechnology Branch

Nicholas Reo
Michael Raymer
Paul Anderson
Andrew Neuforth
Wright State University
Dayton OH

Harry Luithardt
Solutions Labs, Inc.
Cambridge MA

Nestor Tarragona
Tufts Medical School
Boston MA

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Air Force Research Laboratory
711 Human Performance Wing
Human Effectiveness Directorate
711th Human Performance Wing
Biosciences and Protection Division
Applied Biotechnology Branch

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REBECCA GULLEDGE, Work Unit Manager Applied Biotechnology Branch	MARK M. HOFFMAN, Deputy Chief Biosciences and Protection Division Human Effectiveness Directorate 711th Human Performance Wing Air Force Research Laboratory

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DelRaso, N[1]; Reo, N[2]; Raymer, M[3]; Luithardt, H[4]; Tarragona, N[5]; Reitcheck[1]; Mahle, D[1]; Anderson[3], P; Neuforth, A[2]

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[2]Wright State University, Boonshoft School of Medicine, Dept of Biochemistry and Molecular Biology, Dayton OH 45429
[3]Wright State University, Dept of Computer Science and Engineering, Dayton OH 45435
[4]Solutions Labs, 400 Technology Square, Cambridge MA 02139-3583
[5]School of Medicine, Tufts Medical Center, 136 Harrison Ave, Boston MA 02110

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In this study we examined the urinary metabolite profiles from rats following a single exposure to the kidney toxicants D-serine, puromycin, hippuric acid and amphotericin B at various doses, and as a function of time post-dose. In toxicology, such dose-time metabonomics studies are important for an accurate determination of the severity of biological effects, and for biomarker identification that may be associated with toxicity. The metabonomics analysis yielded a dose-response curve in principal component analysis space, and was able to detect exposure to D-serine and puromycin at much lower doses than standard clinical chemistry measures. Additionally, characteristic features in the urinary metabolite profiles could be ascertained as a function of dose. The results showed common features and some unique features in urinary metabolite profiles when analyzed by NMR and LC-MS, respectively.

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VOLUME 4: METABONOMICS

Biomarkers to Liver and Organ Damage

In this study we examined the urinary metabolite profiles from rats following a single exposure to the kidney toxicants D-serine, puromycin, hippuric acid and amphotericin B at various doses, and as a function of time post-dose. In toxicology, such dose-time metabonomics studies are important for an accurate determination of the severity of biological effects, and for biomarker identification that may be associated with toxicity. The metabonomics analysis yielded a dose-response curve in principal component analysis space, and was able to detect exposure to D-serine and puromycin at much lower doses than standard clinical chemistry measures. Additionally, characteristic features in the urinary metabolite profiles could be ascertained as a function of dose. The results showed common features and some unique features in urinary metabolite profiles when analyzed by NMR and LC-MS, respectively.

Keywords: genomics, proteomics, metabonomics, biomarker, metabolite, exposure, liver, kidney, toxicity, gene, expression, nephrotoxin, D-serine, hippuric acid, Puromycin, Amphotericin B, LC-MS, NMR, urinary

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PREFACE

This research was accomplished at the Applied Biotechnology Branch, Human Effectiveness Directorate of the 711th Human Performance Wing (711 HPW/RHPB) of the Air Force Research Laboratory, Wright-Patterson AFB, OH, under Dr. John J. Schlager, Branch Chief. This technical report was written for AFRL Work Unit 7184D405.

All studies involving animals were approved by the Wright-Patterson Institutional Animal Care and Use Committee, and were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, in accordance with the *Guide for the Care and Use of Laboratory Animals* (1996).

1. **INTRODUCTION**

Metabonomics is defined as "the quantitative measurement of the time-related multiparametric metabolic response of living organisms to pathophysiological stimuli or genetic modification" (Nicholson et al., 1999). This term is derived from the Greek roots "meta" (change) and "nomos" (regularity and order); referring to the ability of chemometric models to classify changes in metabolism (Lindon et al., 2004). This biotechnology was pioneered by Jeremy Nicholson, Elaine Holmes and John Lindon in the late "90s" at the Imperial College in London (Nicholson et al., 1999). The field of metabonomics is concerned with the study of fixed cellular and biofluid concentrations of endogenous metabolites, as well as dynamic metabolite fluctuations, exogenous species, and molecules that arise from chemical rather than enzymatic processing (Lindon et al., 2003).

Metabonomics is an approach used to characterize the metabolic profile of a specific tissue or biofluid. Because many biofluids can be easily obtained either non-invasively (urine) or minimally invasively (blood), they are typically used in metabonomic studies. However, other *in vivo* biofluids such as saliva, cerebrospinal fluid, bile, and seminal fluid, as well as *in vitro* biofluids such as cell culture supernatants and tissue extracts, can also be used. Metabonomics is an attractive approach to the study of time-related metabolic responses to pathophysiological processes because biological and chemical agents, or drugs, cause perturbations in the concentrations and fluxes of endogenous metabolites involved in critical cellular pathways. In other words, cells respond to toxic insult or other stressors by altering their intra and/or extracellular environment in an attempt to maintain a homeostatic intracellular environment. This metabolic alteration is expressed as a "fingerprint" of biochemical perturbations that is characteristic of the type and target of a toxic insult or disease process. These metabolic alterations are often seen in the urine as changes in metabolic profile in response to toxicity or disease as the body attempts to maintain homeostasis by eliminating substances from the body (Shockcor and Holmes, 2002). Subtle responses to toxicity or disease under conditions of homeostasis also result in altered biofluid composition. A recent article by Don Robertson (2005) provides an excellent review of the use of metabonomics in toxicology.

A frequent misconception is that metabonomics is based primarily on nuclear magnetic resonance spectroscopy (NMR)-derived data. In theory, any technology that has the capacity to generate comprehensive metabolite measurements can be used for metabonomics. The most

common analytical platforms used today in metabonomics are proton NMR and mass spectroscopy (MS) coupled to liquid chromatography (LC) and gas chromatography. The advantages of NMR-based metabonomics include nondestructive analysis, analysis of intact tissues, molecular structure analysis, and quantitative small-molecule analysis. The non-selectivity, lack of sample bias and reproducibility of NMR (Keun et al. 2002) is of critical importance when considering toxicological screening applications. Changes in NMR-derived urinary metabolite levels have proven to be a sensitive indicator of chemical-induced toxicity (Robertson et al., 2000; Shockcor and Holmes, 2002; Waters et al., 2002; Nicholson, et al., 2002). MS offers the ability to detect chemical classes not detected by NMR (i.e. sulfates), and the capability to detect lower abundance metabolites with little sample processing (Dunn and Ellis, 2005). This is of critical importance if one is searching for novel biomarkers of toxicity or disease. Urinary metabolite analysis using LC-MS has also been used to profile chemical-induced toxicity (La et al., 2005). It is clear to see that MS is complimentary to NMR data and facilitates metabolite identification.

The objective of the present study was to identify organ-selective toxicity biomarkers for two organ systems in a rat animal model. The two organ systems selected were kidney and liver Although some initial work with a liver toxicant (alpha-naphthylisothiocyanate; ANIT) was performed under an earlier protocol (data not shown), the data presented in this final report is concerned with changes in gene transcript, protein and metabolite levels associated with kidney specific toxicity in rats following exposure to rationally selected chemicals using genomic, proteomic and metabonomic profiling techniques. Four chemicals were chosen (D-serine, puromycin, hippuric acid and amphotericin B) that targeted different functional regions of the kidney (i.ie. proximal tubules, distal tubules, glomerulus and medulla). Profile changes, after additional testing and validation, may be proven to be sensitive and reliable biomarkers for acute organ-specific regional toxicity/damage in human population. A summary of the effects of the selected chemicals on the kidney is presented below.

2. METHODS

2.1. Urine Sample Processing

Urine samples for NMR and LC/MS analyses were prepared as described by Robertson et al. (2000) and modified as follows. Urine samples were thawed at 4 °C overnight, and then equilibrated to room temperature just prior to NMR sample preparation. A 600 µL aliquot of urine was transferred to a 1.5 mL Eppendorf tube, mixed with 300 µL of phosphate buffer (0.2 M monosodium phosphate and 0.2 M disodium phosphate, pH 7.4), and allowed to equilibrate for ten minutes. Samples were then centrifuged at 5000 rpm for ten minutes to remove any particulate matter, and 550 µL of supernatant was transferred to a 5 mm NMR tube. An internal standard consisting of 150 µL of trimethylsilylpropionic (2, 2, 3, 3 d_4) acid (TSP) dissolved in deuterium oxide was added at a final concentration of 2 mM.

2.2. 1H-NMR Spectroscopy

Proton NMR spectra were acquired at 25_C on a Varian INOVA operating at 600 MHz. Water suppression was achieved using the first increment of a NOESY pulse sequence, which incorporated saturating irradiation (on resonance for water) during the relaxation delay (7.0 s total; 2 s with water pre-saturation) and the mixing time (50 ms total; 42 ms with water irradiation). Data were signal averaged over 64 transients using a 4.0 s acquisition time and interpulse delay of 11.05 s.

2.3. NMR Multivariate Statistical Analysis

NMR spectral data were processed using Varian software and employing exponential multiplication (0.3 Hz line-broadening), Fourier transformation, and baseline flattening (fifth-order polynomial and spline fitting routines). Spectra were subdivided into 280 regions (bins) of 0.04 ppm width for integration using Varian Binning software. The residual water signal (~4.8 ppm) and the urea signal in urine spectra (~5.8 ppm) were excluded from the analyses. Integrated bin areas will be transferred to an Excel file and normalized to the TSP signal intensity. A second normalization will be performed by summing 256 bins over the entire metabolite range, which excludes the region of TSP signal (0.53 to -0.28 ppm). These 256 'sum normalized' bins will be used as input to principal component analysis (PCA) and linear

discriminant analysis (LDA) performed using MatLab 7.0. Pattern recognition methods (PCA scores plots) will enable data visualization and help to identify interesting samples and timepoints such as: outliers; time of maximum biochemical impact; average time to recovery; rats showing different rates of recovery; etc. OPLS-DA will enable classification into specific groups. The OPLS correlation coefficients will then be used to weight and rank the salient spectral peaks (metabolites signals). Various quality metrics will be used to validate and provide a level of statistical confidence to the results (i.e, Q2, R2, accuracy, leave-one-out cross validation, bin-by-bin t-tests, etc). Bin-by-bin t-tests will identify significant spectral features that show similar patterns of change across the study time-course. Statistical group comparisons and hierarchal cluster analysis will be conducted to discern similar and dissimilar spectral features between exposed and control animals. Data from individual animals will be analyzed as a function of time, as each animal can serve as its own control. Comparisons between animals will help to identify metabolites profiles as markers of infection or resilience to infection. The salient spectral resonances will be assigned to metabolites using Chenomx 5.1 software, on-line NMR databases (i.e., mmcd.nmrfam.wisc.edu; U Wisc, etc), and by "spiking" samples with known compounds, if necessary.

2.4. LC/MS Metabonomics

Collected urine will be centrifuged on a bench top Eppendorf centrifuge for 15 minutes at 14.5k rpm top remove particulate matter. After centrifugation samples will be filtered through a PALL acrodisk. Metabolite separation will be completed using a Waters Ultra High Performance Liquid Chromatography system (UPLC). Five microliters of each sample will be injected onto an Acquity UPLC BEH C18 column (2.1 x 100 mm). Water with 0.1% formic acid will be used as mobile phase A and ACN with 0.1% formic acid will be used as mobile phase B. The gradient will be increased from 2% Buffer B to 98% Buffer B over 11 minutes followed by re-equilibrating the column with 2% Buffer A. The UPLC is coupled to a Micromass Quadrupole-Time of Flight (Q-ToF) mass spectrometer (Waters, Milford, MA). The electrospray conditions will be as follows: capillary voltage, 3200; sample cone voltage, 35.0; extraction cone voltage, 1.5; desolvation temperature, 320°C; and the source temperature, 130°C: nitrogen as the cone gas. Survey scan data will be acquired in centroid mode from 80-1000 m/z with a scan time of 0.4s and an inter-scan delay of 0.1s. For accurate mass measurements, a reference

compound, leucine enkephalin (Sigma-Aldrich, St. Louis, MO) will be injected periodically using a lockspray source. The lockspray signal is set to acquire every 720s and two scans are averaged for each run. Setting the lockspray as above allows data collection within 20ppm without losing any data for subsequent data analysis. The conditions were extensively tested in the RHPB mass spectrometry laboratory. Samples will be run in duplicate in both positive and negative modes and later analyzed using in-house developed software to determine peaks of interest.

2.5. LC/MS Data Analysis and Metabolite Identification

Full (survey) scan data will be subjected to data analysis using software package developed in our laboratory. Our bioinformatic platform allows us to identify and statistically access metabolite features that exhibit significant change between sample groups. The package incorporates proprietary algorithms for nonlinear retention time alignment, peak detection and peak matching. Complete differential analysis and visualization tools as well as multidimensional feature filtration tools are integrated into the software. Numerous statistical tests including 2-way Analysis of Variances, Principal Component Analysis etc. are incorporated into the package. The software can identify and remove on demand all minor monoisotopic peaks that are related to the major monoisotopic peaks. It is also capable of searching and reporting any peaks that exhibit adduct acquisitions or water losses. Only features with p values less than 0.05 will be considered for further analysis. Peaks that pass at least 2 fold change filter as well as set statistical criteria will be selected for identification of metabolites related to the corresponding peaks.

To acquire MS/MS fragmentation data, samples will be run under the same LC conditions as the original samples. Data will be acquired using data dependent acquisition method. The Q-ToF instrument will be set to automatically switch on up to three ions in each survey scan whose intensity exceeded 150 counts/second. Survey data will be acquired with a scan time of 1.0s and an inter-scan delay of 0.1s. MS/MS data is acquired for each precursor ion for up to 10s or until signal decreased below 30 counts/second with a scan time of 0.5s and an inter-scan delay of 0.1s. Argon will be used as the collision gas. Centroid data is collected from 50 to 750 m/z. Once MS/MS data for peaks of interest is collected preliminary search will be conducted in existing databases that are available to the researchers. These will include the following databases: Metlin (Scripps Center for Mass Spectrometry, The Scripps Research

Institute), KEGG Compound (Kanehisa Laboratories, Kyoto University and the Human Genome Center, University of Tokyo) and Human Metabolome Project (Alberta, Canada). When matches are found standards will be purchased from commercial sources and run at 1 mg/ml (1µg injection) under the same LC conditions as the samples. The same data dependent acquisition method as for MS/MS fragmentation data acquisition will be used to run the standards. We currently have a collection of 51 metabolite standards in our laboratory at RHPB. The collection is expected to grow to several hundred within next 3 months alone. Retention time and MS/MS fragmentation data match between commercial standard and metabolite selected in the profiling will confirm metabolite identification.

If we do not find any match in the existing databases the peaks of interest will be further investigated using Linear Ion Trap -Fourier Transform mass spectrometer (LTQ-FT). LTQ-FT is a high mass accuracy instrument with an accuracy routinely lower than 1ppm even for higher mass range compounds (>250 m/z). For compounds less than 250 m/z the mass accuracy is around 50ppb. Mass accuracy advantage allows deducing elemental composition of the peak of interest. Peaks of interest will be subjected to MS/MS fragmentation. Optimum conditions will be used to acquire MS/MS fragmentation data. Particularly, different energies to fragment ions of interest will be investigated using both Collision Induced Dissociation and Pulsed Q Dissociation. Based on the elemental composition standards will be purchased from commercial sources. In case a standard is not commercially available possible compounds within determined elemental composition with structural considerations from fragmentation data will be synthesized. MS/MS fragmentation data under the original LC conditions will be collected for synthesized compounds. Metabolite identification will be confirmed when retention time and MS/MS fragmentation data match between synthesized compound and metabolite selected in the profiling.

3. RESULTS AND DISCUSSIONS

3.1. D-Serine NMR

3.1.1. NMR Spectroscopy of Urine Samples

The representative NMR spectra shown in Figure 1 show the changes in endogenous components of urinary metabolites observed 24 hours post-exposure in rats given a single i.p. dose of normal saline (Figure 1A) and 500 mg/kg D-serine (Figure 1B), respectively. The NMR instrument phasing and baseline correction of the data was not used to generate the spectra in Figure 1. Instead, the automatic data processing procedure of the Chenomx (Chenomx Inc., Edmonton, Canada) NMR Processor application was implemented. In order to visualize the data, the NMR traces were binned with bin sizes of 0.0005 ppm (i.e. full resolution), and the data was scaled using the area of the peak associated with the internal standard (i.e. TSP). Water and urea resonances were removed from the NMR traces. The pre-dose urine NMR spectrum is representative of naïve animals. Visual inspection of the NMR spectra twenty-four hours following exposure to 500 mg/kg D-serine clearly reveals that the intensities of resonances from a number of metabolites were clearly altered following exposure to D-serine. The pre-dose and 24 hour post-dose data of animal 251 were examined.

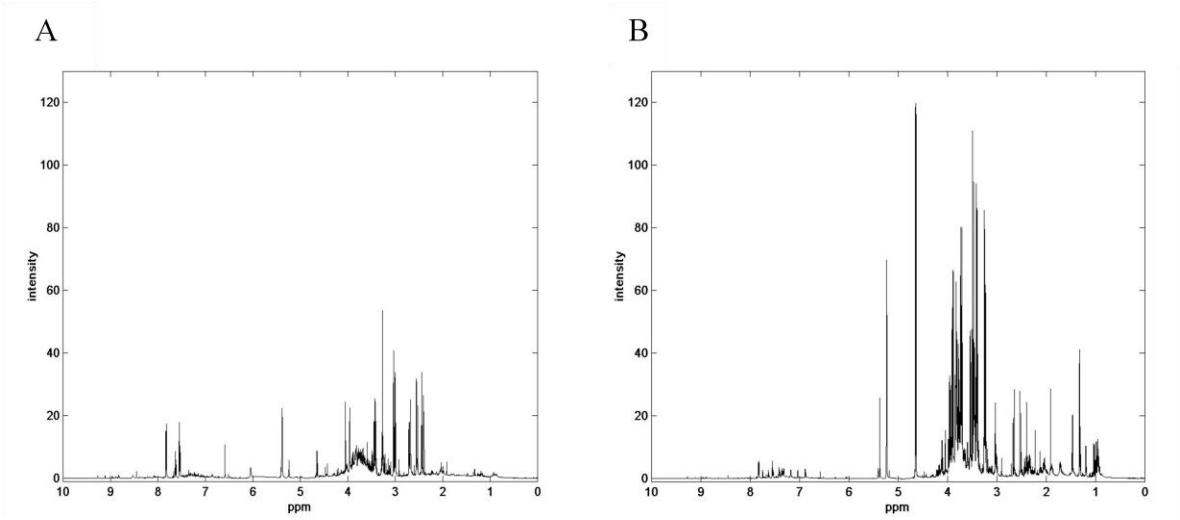


Figure 1: Representative 600 MHz ^{1}H NMR dosed rat urine spectra

A) Normal saline control and B) 500 mg/kg D-serine. Peaks have been scaled using area of TSP internal standard.

3.1.2. NMR PCA Results

The 256 bin intensities from each urine NMR spectrum provided multivariate data input to PCA, which was conducted for each dose group in combination with the vehicle-treated (normal saline) control group. The results are presented graphically in Figure 2.

Figure 2 shows PCA scores plots (PC1 vs. PC2) for each dose group versus controls as a function of time from pre-dose (d0) to day-4 post-dose (d4). The use of the first two principal components (PCs) was sufficient to demonstrate pattern separation between treatment groups. For all analyses, the first two PCs explained ~70% of the variance in the data. Data points represent the mean values for the PCs at each time point, and the ellipse encircling each point depicts the ±standard error (SE) boundaries. We chose to plot only the mean values because graphing all sample points results in plots that are cluttered and difficult to interpret, especially for the lower dose groups, which show little separation (Figure 2B). The plots in Figure 2 show differences in the mapping positions reflecting changes in biochemical composition of the urine between control and D-serine treated animals.

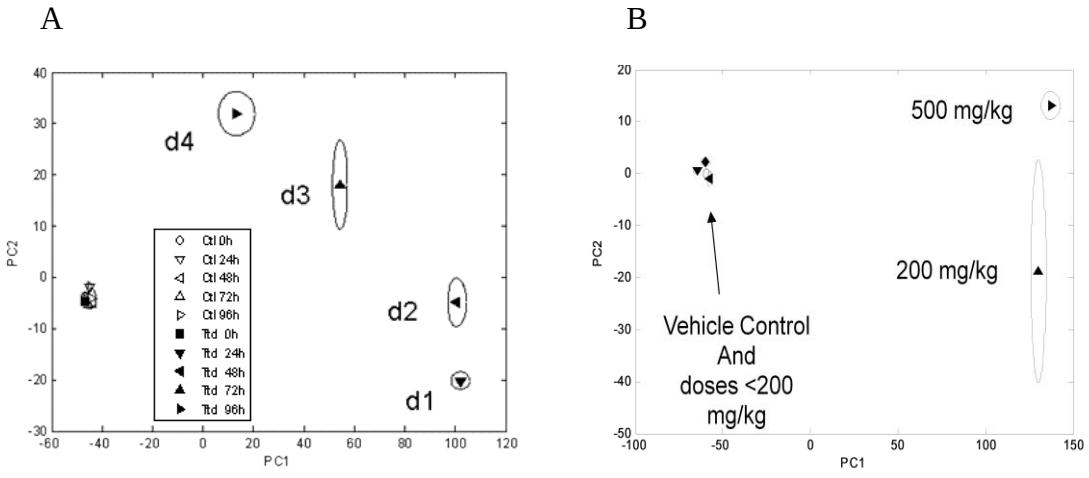


Figure 2: PCA analysis of urinary metabolites from rats exposed to D-serine as a function of dose and time

Treated (filled symbols) and vehicle-treated (saline) control (open symbols) data are plotted as the mean value for the first two principal components (PC1 vs. PC2) with an ellipse indicating the ±SE in both dimensions. (A) PCA analysis of the effects of D-serine (500 mg/kg) over time from day-0 (pre-dose) to day-4 (d4) post-dose. (B) PCA analysis of rat urinary metabolites as a function of dose (5 – 500 mg/kg) at d2 post-dose.

In general, urine NMR spectra of animals least affected by D-serine exposure mapped in the general vicinity of coordinate clusters relating to vehicle control. Clear separation between control and D-serine-treated animals was observed 24 hours (d1) post-dose and remained separated out to four days post-dose. However, the trajectory of separation appeared to moving back toward pre-dose over time following maximal separation between day-1 and day-2 (Figure 2A).

The PCA plots shown in Figure 2 indicate that the maximal dose-response effect of D-serine on rat urinary metabolites is observed at approximately day-2 post-dose. Therefore, a dataset for all treatment groups was constructed using only the day-2 urine NMR data and a PCA analysis was performed as a function of dose. The scores plot (PC1 *vs*. PC2) depicted in Figure 2B shows a clear separation of groups in PC space as a function of dose with doses $\geq$ 200 mg/kg. All doses less than 200 mg/kg were found to co-locate with control in PC space.

3.1.3. Chenomx D-Serine Profiling

After the NMR spectra were created from the FID data, the Chenomx NMR profiler was used to bin the data with 0.04 ppm bins prior to multivariate analysis. Water and urea resonances were deleted, and the binned spectra were normalized. Only the region between 0 and 10 ppm was retained, and each binned spectrum contained 234 bins. As a first assessment of group separation, the data matrix was mean centered, and Pareto scaled prior to a PCA analysis. This analysis showed that most of the data variation resided in the first component.

In order to identify important urinary metabolites and possible biomarkers of kidney toxicity, the Chenomx NMR Profiler application was initially applied to one sample selected from the saline controls and one sample selected from the high-dose D-serine treatment group (500 mg/kg). These samples were chosen based on their large degree of separation in the PCA

scores plot (Figure 2). Two more samples were then profiled in order to assess whether the qualitative conclusions drawn from examining the first pair were also exhibited in the second pair. Pair-wise analysis was found to be consistent, and therefore, the spectra from the initial pair of urine samples were annotated in detail (Figure 4).

Based on a collaborative agreement between Solutions Labs (Cambridge, MA) and Chenomx Inc., Chenomx profiled NMR samples from the D-serine study conducted by Air Force Research Laboratory at Wright Patterson AFB, Dayton, OH, for the purpose of developing an optimal analysis protocol for the AFRL samples. Solutions Labs supplied Chenomx with phased and baseline corrected Varian formatted proton NMR rat urine data (supplied by Dr. Reo, Wright State University) from a control sample and from a D-serine (500 mg/kg) exposure sample collected at 24 hours post-exposure. Since this dose of D-serine and exposure time was expected to induce maximal metabolic perturbations, a significant alteration in urinary metabolite profile was observed that was similar to that previously identified in the literature (Williams et al., 2003). Representative metabolites identified by Chenomx are summarized in the Table 1.

Table 1: Preliminary identification and concentration estimates of the un-normalized data from rat urine of pre and post (24 hour) dosing (500 mg/kg D-serine).

Index	Metabolite	0 h Predose (mM)	24 h D-serine (500mg/kg) (mM)
1	1-Methylnicotinamide	0.48	0.35
2	2-Oxoglutarate	7.44	1.80
3	3-Hydroxybutyrate	0.22	1.90
4	3-Hydroxyisovalerate	0.07	0.37
5	Acetate	0.44	2.95
6	Acetone	0.13	1.03
7	Alanine	0.23	4.07
8	Allantoin	8.67	1.70
9	Anserine	0.31	0.07
10	Betaine	0.33	1.80
11	Citrate	6.75	8.68
12	Creatine	0.22	1.22

13	Creatinine	3.29	2.33
14	Dimethylamine	0.69	0.26
15	Formate	0.64	0.28
16	Fumarate	0.15	0.07
17	Glucose	4.90	62.66
18	Glycerate	1.78	2.11
19	Hippurate	5.25	1.28
20	Histidine	0.66	1.20
21	Isoleucine	0.07	1.14
22	Lactate	0.36	6.13
23	Leucine	0.22	1.82
24	Malate	1.24	1.64
25	Mannose	0.19	1.55
26	Methionine	0.09	0.32
27	Methylamine	0.37	0.15
28	Methylmalonate	0.14	0.03
29	Methylsuccinate	0.09	0.32
30	"N,N-Dimethylglycine"	0.21	0.26
31	N-Acetylaspartate	0.33	0.42
32	NAD+	0.03	0.00
33	O-Acetylcarnitine	0.11	0.36
34	Phenylalanine	0.39	1.35
35	Proline	1.46	3.26
36	Pyruvate	0.14	0.81
37	S-Adenosylhomocysteine	0.03	0.00
38	Serine	1.36	14.87
39	Succinate	1.32	1.33
40	Sucrose	1.39	1.44
41	Trigonelline	0.56	0.17
42	Trimethylamine N-oxide	1.17	0.71
43	Tryptophan	0.29	0.39
44	Tyrosine	0.23	1.02
45	Uracil	1.12	0.37
46	Urea	64.21	19.69
47	Uridine	0.10	0.12
48	Valine	0.09	1.90

49	Xanthine	0.21	0.62
50	trans-Aconitate	3.17	0.72

Discussions between Solutions Labs and Chenomx took place with regards to optimal approaches to utilizing the Chenomx's NMR Profiler database for profiling rat urine data. One recommendation was to attempt a fit of each sample to a list of 84 metabolites that Chenomx technology developers had established to be the most easily identified in urine. Chenomx's opinion was supported by their experience in profiling several hundred urine samples for various clients. The list of these metabolites is presented in Table 2 due to the likelihood that many of the potential kidney toxicity biomarkers will ultimately be on this list.

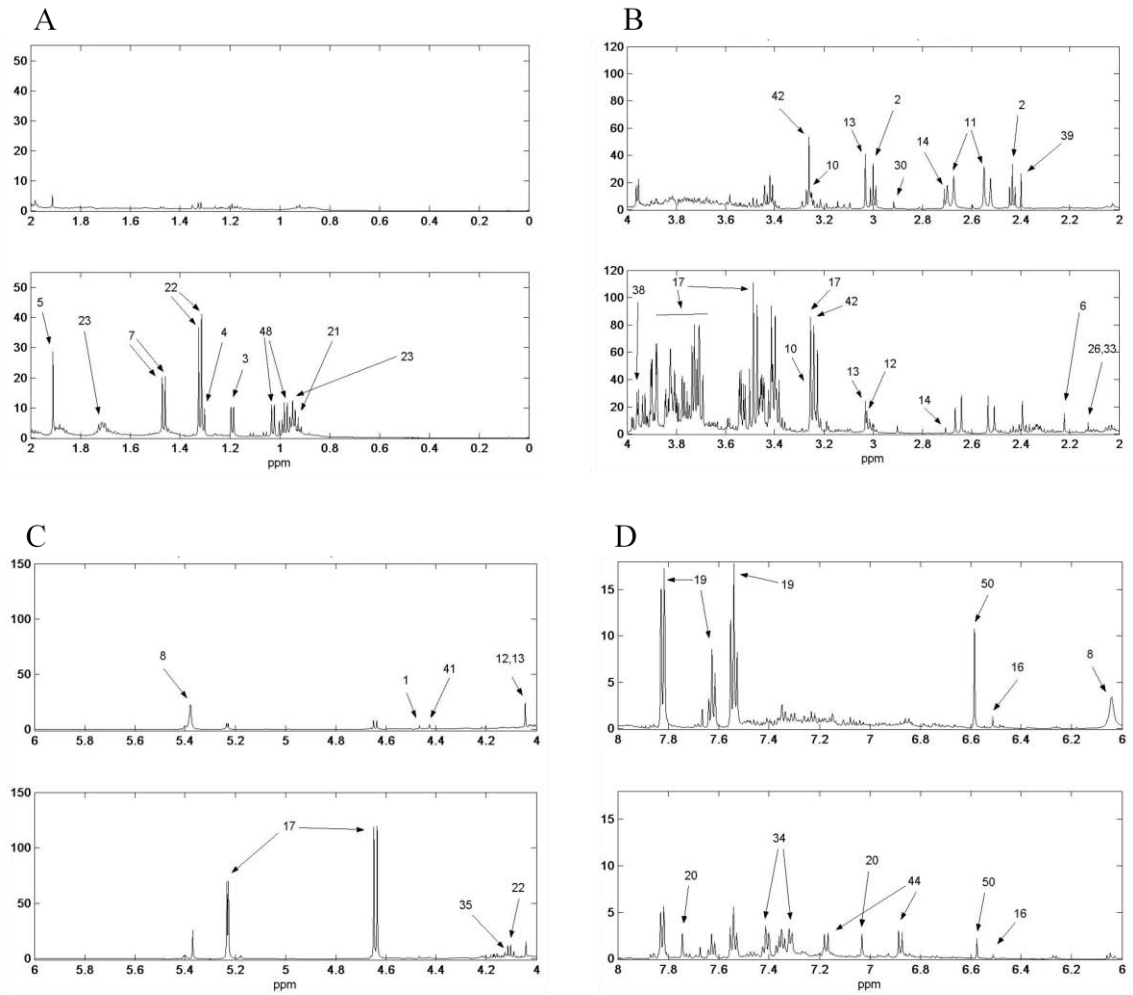
Table 2: List of the most easily identified urinary metabolites

Metabolite list was developed by Chenomx using their NMR Profiler 5.0 software.

1,6-Anhydro-β-D-Glucose	Carnitine	Histidine	Pyruvate
1-Methylnicotiamide	Choline	Hypoxanthine	Salicylurate
2-Hydroxyisobutyrate	Cis-Aconitate	Imidazole	Serine
2-Oxoglutarate	Citrate	Indole-3-Acetate	Suberate
3-Hydroxybutyrate	Creatine	Isoleucine	Succinate
3-Hydroxyisovalerate	Creatinine	Isopropanol	Sucrose
3-indoxylsulfate	D-Glucuronate	Lactate	Tartrate
4-Hydroxyphenylacetate	DMA	Leucine	Taurine
6-Hydroxynicotinate	Ethanol	Lysine	Threonine
Acetate	Ethanolamine	Malonate	TMA
Acetoacetate	Formate	Methanol	TMAO
Acetone	Fucose	Methylsuccinate	Trans-Aconitate
Adipate	Fumarate	N,N-dimethylglycine	Trigonelline
Alanine	Glucose	N-Acetylglycine	Tryptophen
Allantoin	Glutamate	O-Acetylcarnitine	Tyrosine
Arginine	Glutamine	O-Phosphocholine	Uracil
Asparagine	Glycerophosphocholine	Oxalacetate	Urea
Aspartate	Glycine	Phenylacetylglycine	Valine
Benzoate	Glycolate	Phenylalanine	Xylose
Betaine	Guanidinoacetate	Propyleneglycol	π-Methylhistidine
Butyrate	Hippurate	Pyroglutamte	τ-Methylhistidine

Based on the indexed metabolite numbers from Table 2 above, annotated NMR tracings at 2 ppm intervals is presented below in Figure 3. These NMR tracings were obtained using

Chnomx Profiler analyses of un-normalized rat urine data derived from control and D-serine treated animals. It is apparent from these annotated tracings that a number of metabolites are differentially regulated.



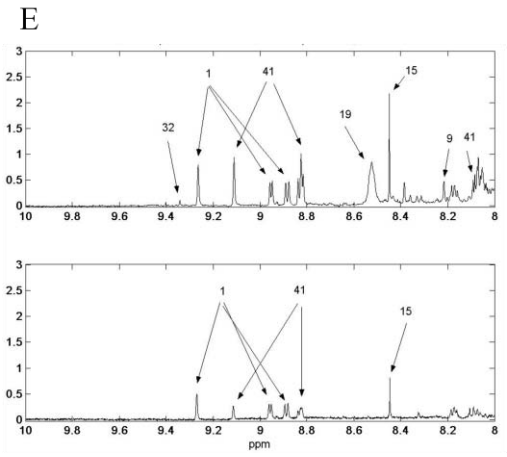


Figure 3: Representative annotated NMR traces (A – E) of predose (top pannel) and 24 hours D-serine post-dose (bottom pannel) rat urine.

The traces of the two samples are in megahertz frequency intervals of 2ppm. A, 0 – 2 ppm; B, 2 – 4 ppm; C, 4 – 6 ppm; D, 6 – 8 ppm and D, 8 – 10 ppm. The number index labels of the figure correspond to metabolite numbers identified in above table. Signal was scaled using area of internal standard peak.

3.1.4. Preliminary Investigation of Candidate NMR Biomarkers of D-serine Exposure

Metabolites were identified as being significantly regulated based on partial least squares-discriminant regression analysis (PLS-DA). Only metabolites with "p" values ≥ 0.05 were considered significantly altered metabolite concentrations measured in the urine of D-serine treated rats when compared to control rat urine (Table 3). Up-regulated metabolites were found to fall-into compound classes generally identified as carbohydrates (i.e. glucose and manose), amino acids (i.e. leucine, histidine, phenylalanine, ect.), ketone bodies (i.e. 3-hydroxybutyrate and acetone). Interestingly, the osmolyte betaine was found to be significantly elevated. This metabolite has been show to be involved in protecting tissue damage in the kidney through methylation (McGregor et al., 2002). In a renal context, betaine is usually considered only in its role as an osmolyte in protection of renal cells from the extreme osmolar environment of the kidney (Burg, 1995). Down-regulated metabolites were found to fall-into compound classes generally related to energy production (i.e. 2-oxoglutarate fumarate and succinate), nicotinate

metabolism (i.e. NAD+, 1-methylnicotinamide, and trigonelline) and osmolytes (i.e. dimethylamine and methylamine).

Table 3: Differentially regulated metabolites in urine of rats exposed to D-serine (500 mg/kg)

Metabolite concentrations considered significantly altered if PLS-DA "p" values ≤ 0.05 when compared to controls (see Table 2).

Up-Regulated	Down-Regulated
3-Hydroxybutyrate	1-Methylnicotinamide
3-Hydroxyisovalerate	2-Oxoglutarate
Acetate	Trans-Aconitate
Acetone	Allantoin
Alanine	Anserine
Betaine	Creatinine
Glucose	Dimethylamine
Histidine	Formate
Isoleucine	Fumarate
Lactate	Hippurate
Leucine	NAD+
Phenylalanine	Succinate
Proline	Trigonelline
Pyruvate	
Tyrosine	
Valine	

Additional rat urine samples from the D-serine study were made available to Chenomx for analysis (i.e. 3 controls and 3 D-serine treated (500 mg/kg). However, no manual parameter adjustments to improve the automatic processing functions of the Chenomx software were implemented. NMR .fid files sent to Solutions Labs were processed as follows prior to being sent to Chenomx: (i) automatic phasing; (ii) baseline correction (with cubic spline option); (iii) line

broadening (with broadening constant 0.50); and (iv) reference deconvolution (using TSP satellite peaks). Because the outputs are automatically normalized, the water resonance (4.7 to 4.9 ppm) and the urea resonance (5.5 to 6.0 ppm) were removed prior to binning and normalization. According to Chenomx documentation, correct phasing and baseline corrections are extremely important for implementing the reference de-convolution, and that procedure in turn may ease subsequent compound profiling significantly.

Urine from control (n = 3) and D-serine exposed rats (n = 3) were re-analyzed using Chenomx's updated profiling software and corrected pre-processed NMR data. Profiles obtained from Chenomx were used to derive estimates of urinary metabolites using the newly released Profiler V.5.0 software. The results of this new profiling analysis are presented below (Table 4). Urine samples from three controls at 24 hours and three high dose D-serine exposed rats at 24 hours are shown. Metabolite concentrations were un-normalized and given in units of mM.

Table 4: Preliminary identification and concentration estimates (mM)

The un-normalized data from rat urine of pre (control, n = 3) and 24 hours post dosing (500 mg/kg D-serine, n = 3).

Metabolite	Control			D-serine		
1-Methylnicotinamide	0.7319	0.7216	0.6602	0.4025	0.2314	0.3996
2'-Deoxyinosine	0.026	0.0056	0.0051	0.0046	0.0203	0
2-Aminoadipate	0.1132	0.032	0.0419	0.0923	0.0616	0.0883
2-Hydroxyisobutyrate	0.0279	0.0214	0.0226	0.0258	0.014	0.0224
2-Oxoglutarate	5.251	5.6185	6.1093	2.0756	1.2933	1.7167
2-Oxoisocaproate	0.0529	0.0586	0.0586	0.0874	0.0548	0.0666
3-Hydroxybutyrate	0.0708	0.075	0.0708	1.2157	0.2693	0.9488
3-Hydroxyisovalerate	0.0303	0.0332	0.0303	0	0	0
3-Indoxylsulfate	0.3569	0.33	0.33	0	0	0
4-Hydroxyphenylacetate	0.2166	0.192	0.192	1.226	1.1882	1.5072
Acetate	0.8086	0.2884	1.0817	2.0534	2.9704	2.9079
Acetoacetate	0.0481	0.0398	0.0785	0.4357	0.1519	0.1685
Acetone	0	0	0	0.7679	0.2982	0.6553

Alanine	0.0941	0.0892	0.1045	3.7773	3.7194	4.9508
Allantoin	12.7514	12.0042	13.5025	6.0297	5.468	5.6138
Benzoate	0	0	0	0.0849	3.2353	0.2637
Betaine	0.2992	0.2904	0.2948	1.5231	2.1485	2.063
Choline	0.0705	0.0705	0.083	0.1913	0.2074	0.2441
Citrate	7.3211	9.4605	11.6852	4.9865	2.4973	4.0095
Creatine	0.0844	0.0978	0.0844	1.1414	0.4076	0.9217
Creatinine	3.7313	3.3006	3.4449	1.7703	1.322	1.7858
Cystine	0	0	0	0.6337	0.5101	0.6337
Dimethylamine	0.664	0.6099	0.6743	0.2653	0.2248	0.2699
Formate	0.743	0.5985	0.7848	0.4153	0.3638	0.4366
Fumarate	0.0797	0.0858	0.1034	0.0633	0.0598	0.07
Glucose	2.973	3.2367	4.0821	81.3264	70.8323	108.6358
Glycine	0.3225	0.2802	0.2802	3.2797	4.8449	3.955
Hippurate	5.9778	7.2902	7.198	2.5598	0.2679	2.8968
Histidine	0.1352	0.0236	0.0262	0.6037	0.9411	0.9077
Isobutyrate	0.0179	0.0179	0.0163	0.1235	0.0724	0.1282
Isoleucine	0	0	0	0.9474	0.8027	1.2884
Lactate	0.4465	0.2273	0.4143	4.4884	6.0789	5.8896
Lactose	0.6302	1.4286	1.0426	0	0	0
Leucine	0.0284	0.0284	0.024	1.6128	1.4276	1.8729
Lysine	0	0	0	2.6145	2.4236	2.5699
Malate	0.4428	0.2888	0.2521	0	0	0
Maleate	0.0371	0.0229	0.0214	0.085	0.0074	0.0494
Malonate	0.6088	0.8019	0.846	0	0	0
Mannose	0.0963	0.1762	0.1342	1.2404	0.805	1.2227
Methionine	0.0286	0.0286	0.0256	0.8303	0.6883	0.9104
Methylamine	0.272	0.272	0.268	0.0884	0.0633	0.0647
Methylsuccinate	0.0302	0.0381	0.0381	0.0314	0.027	0.0344
N,N-Dimethylglycine	0.2339	0.2672	0.2962	0.3403	0.2359	0.2863

O-Acetylcarnitine	0	0	0	0.1394	0.058	0.0842
O-Phosphocholine	0.1575	0.096	0.0818	0.2639	0.1549	0.2983
Oxalacetate	0.4913	0.5317	0.4887	0	0	0
Phenylacetylglycine	0.5965	0.5593	0.5593	0.6983	0.0948	0.6793
Phenylalanine	0	0	0	1.0252	0.9477	0.9748
Proline	0	0	0	1.6814	2.541	2.541
Pyruvate	0.0952	0.0952	0.0916	0.9871	0.8349	0.9866
Serine	0	0	0	18.3142	11.7245	19.9286
Succinate	1.4397	1.6667	1.6191	1.0249	0.839	1.1301
Sucrose	0.3249	0.4614	0.9251	1.0818	1.5891	2.4194
Taurine	5.928	5.928	4.5552	0	0	0
Threonine	0.0361	0.0349	0.0326	3.3631	3.1769	3.9121
Trigonelline	0.5273	0.5143	0.556	0.1902	0.199	0.237
Trimethylamine	0.0103	0.0044	0.0083	0.0217	0.0042	0.0171
Trimethylamine N-oxide	0.7264	0.6534	0.6901	0.3612	0.2431	0.3124
Tryptophan	0	0	0	0.2498	0.2687	0.2895
Urea	61.523	67.9166	89.3157	45.9744	27.9703	48.1148
Valine	0.024	0.0234	0.024	2.1066	1.8604	2.4504
Xylose	0.1605	0.1589	0.1445	0	0	0
cis-Aconitate	0.9291	0.7713	0.7642	0.3765	0.2875	0.3368
trans-Aconitate	1.6975	2.1215	2.5865	0.6384	0.4196	0.8649
trans-4-Hydroxy-L-proline	0	0	0	0.7686	0.6686	0.7932

Although metabolites identified using Chenomx's updated Profiler 5.0 software were similar to that identified using their earlier software version (see Table 1), additional metabolites were identified (see Table 4; i.e. 2-aminoadipate, acetoacetate, choline, cystine, lactose, etc.). However, the concentrations of metabolites identified in Table 1 were only derived from one control and one D-serine treated (500 mg/kg). When the average metabolite concentration from

three animals were used, some of the previous identified metabolites that were identified as being significantly differentiated (i.e. PLS-DA "p" value ≤ 0.05) in Table 3 were shown not to meet the "p" value criteria (see Table 5). Furthermore, the updated Chenomx Profiler 5.0 software identified new metabolites that were significantly differentially regulated, and failed to identify some previously identified metabolites when compared to its earlier Profiler software.

Table 5: Potentially differentially regulated metabolites in urine of rats exposed to D-serine (500 mg/kg)

Metabolite concentrations considered significantly altered if the average concentration (n = 3) were ≥ 2-fold the average at pre-dose (see Table 9).

Up-Regulated	Down-Regulated
3-Hydroxybutyrate	3-Hydroxyisovalerate
4-Hydroxyphenylacetate	3-Indoxylsulfate
trans-4-Hydroxy-L-proline	1-Methylnicotinamide
O-Acetylcarnitine	2-Oxoglutarate
Acetate	cis-Aconitate
Acetone	trans-Aconitate
Acetoacetate	Allantoin
Alanine	Citrate
Benzoate	Creatinine
Betaine	Dimethylamine
Choline	Hippurate
Creatine	Lactose
Cystine	Malate
Glucose	Malonate
Glycine	Methylamine
Histidine	Oxalacetate
Isobutyrate	Succinate
Isoleucine	Taurine

Lactate	Trigonelline
Leucine	Trimethylamine N-oxide
Lysine	Xylose
Manose	
Methionine	
Up-Regulated	**Down-Regulated**
O-Phosphocholine	
Phenylalanine	
Proline	
Pyruvate	
Serine	
Sucrose	
Threonine	
Trimethylamine	
Tyrosine	
Tryptophan	
Valine	

3.2. D-Serine LC/MS

3.2.1. LC/MS Spectroscopy of Urine Samples

Acquisition of the LC-MS positive ionization data was obtained from the Laboratory of Translational Medicine at Harvard Medical School for D-serine metabonomics. The preprocessed data (Waters MarkerLynx output) was transferred to Solutions Labs for preliminary analysis. Data was acquired on the Waters LCT Premier metabonomics platform using the previously described sample preparation and chromatography. Data acquisition was restricted to masses in the interval of 80 to 850 Daltons for species restricted to retention times between 0 and 10 minutes. The data acquisition protocol involved stepping through the experimental sample

acquisition times in sequence, but at fixed times (e.g. 24 hour samples), data acquisition was alternated between groups so that for the fixed time, statistical biases due to instrumental drift are eliminated.

After data acquisition, the D-Serine data files were individually processed by MarkerLynx which yielded a large number of variables (e.g. 35 thousand for D-serine). The data tables were filtered by imposing a requirement that variables would be eliminated from consideration unless they had non-zero intensities for at least 80% of the pre-dose samples. Initially, differential regulation was considered only in variables that were either endogenous metabolites or a consequence of normal rat diet. In follow-up analysis, other filterings were imposed that might include toxin-induced metabolite expression that were below detection limit in pre-dose animals.

3.2.2. LC/MS PCA Analysis

After variable filtering, the data matrix was modified by replacing the remaining zero readings with a small cut-off estimated as half the lowest non-zero intensity. The remaining data transformation included intensity sum normalization and variable scaling to unit variance. PCA analysis was first used to determine the effects of D-serine exposure on rats with respect to dose (Figure 4). To assess the degree to which toxic response alters the metabolome of exposed animals, the time-dependent metabolic profiles of the highest dosed D-serine group (500 mg/kg) was investigated using PCA analysis of data matrices which included only the control subjects and the high dose subjects (Figure 5).

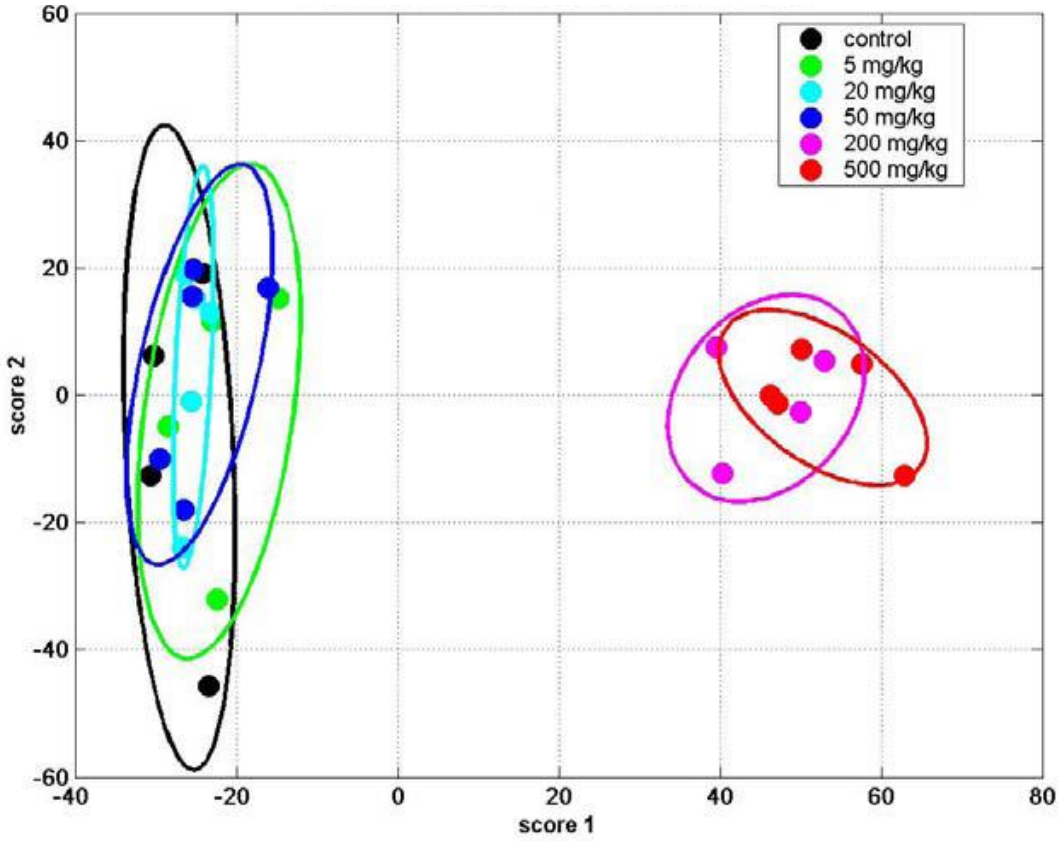


Figure 4: PCA scores analysis for the D-serine data acquired at 24 hours post-dose for the exposure groups of the study (controls, 5, 20, 50, 200, and 500 mg/kg)

The spectrum of eigenvalues indicated that the first component captures almost an order of magnitude more variance than any other component. If it can be shown that the associated scores are associated with the toxic perturbation, then this result would imply that the toxicity leads to readily measurable changes in the metabolome. Indeed, a scores plot of the first two components reveals that the first component is associated with the effect of the administration of the D-serine (Figure 5). The second component, associated with a much smaller variance, appears to capture the effects of the saline injection and the effect of instrumental drift during data acquisition. Overall, the highest D-serine dosed group (500 mg/kg) strongly clustered away from the controls, and this will allow for identification of plausible biomarker candidates. Moreover, the rapid response to D-serine is consistent with reports from the literature (Williams et al., 2003).

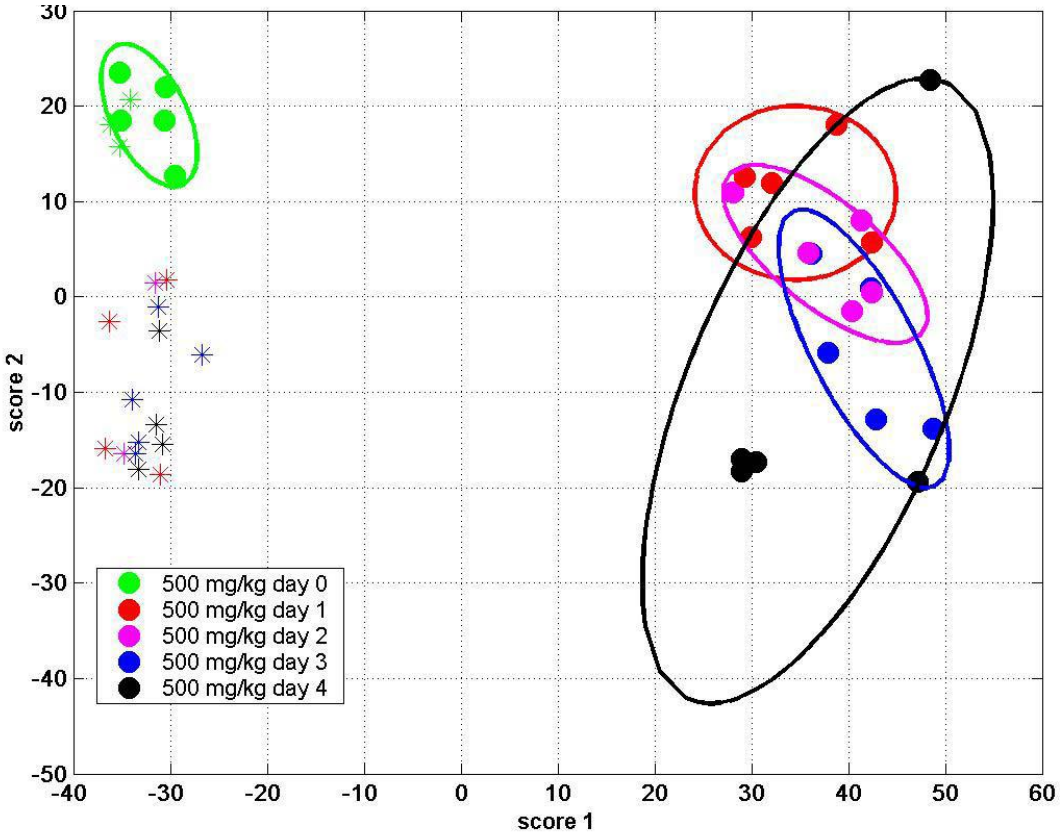


Figure 5: Scores plot for the first two PCA components of the D-serine data.

The points plotted with stars (*) are the control group whose color-coding matches that of the dosed group. Post-dosed 500 mg/kg animals clearly cluster away from their pre-dosed state and away from controls.

3.2.3. Preliminary Candidate LC/MS Biomarkers of D-serine Exposure

Analysis of group separation was extended to attempt to identify compounds that were indicative of D-serine exposure. As indicated previously, initial efforts were restricted to a data matrix that was created by filtering out compounds that were not present in at least 80% of the pre-dose rat population. This procedure was referred to as "endogenous metabolite filtering." Solutions Labs constructed a mass database using sources such as KEGG and Metlin to allow an automated search for measured masses around windows of specified width (plus or minus 20 ppm). Application of an automatic search yielded a large number of potential identifications (over 4000), and most of these were false positives. In order to resolve this problem, Solutions Labs has developed a retention time database for making more precise identifications. Currently,

this database contains about 60 compounds, but some interesting metabolite identifications are already possible. With an expanded metabolite retention library, Solution Labs expects to be able to extract far more information from their existing data sets. The assigned identity of potential biomarkers was made using the metabolite library (Table 6). Of the metabolites listed, the assignment of proline is the most uncertain due to the magnitude of its deviation with its library retention time. A follow-up investigation would be necessary to resolve this.

Table 6: Potential biomarker candidates tentatively identified using LC/MS

Results of applying automated mass identification along with retention time library data to make tentative identifications of variables in the D-Serine data set that was filtered to include only variables expressed consistently across pre-dose animals.

Name	mass tabulated	mass measured	mass difference	Retention Time	RT Library
Tryptophan	204.0899	204.0889	0.0010	3.169	3.11
Phenylalanine	165.0790	165.0781	0.0009	2.471	2.50
5-Hydroxytryptophan	220.0848	220.0868	0.0020	2.174	2.15
Leucine	131.0946	131.0946	0.0000	1.957	1.98
5-Oxo-D-proline	129.0426	129.0428	0.0002	1.455	1.56
Xanthine	152.0334	152.0359	0.0025	1.542	1.41
Citrate	192.0270	192.0277	0.0007	1.259	1.31
4-Guanidinobutanoate	145.0851	145.0854	0.0003	1.006	1.11
AMP	347.0630	347.0682	0.0052	0.697	0.88
Adenine	135.0545	135.0568	0.0023	0.944	0.87
Creatine	131.0695	131.0703	0.0008	0.697	0.74
Proline	115.0633	115.0651	0.0018	1.004	0.73
Maltose	342.1162	342.1212	0.0050	0.857	0.72
Thiamin	264.1050	264.1016	0.0034	0.678	0.67
Creatinine	113.0589	113.0591	0.0002	0.674	0.67
Betaine	117.0790	117.0799	0.0009	0.646	0.67
Histidine	155.0695	155.0719	0.0024	0.670	0.61

The significance of all variable metabolites as potential biomarkers was investigated in the context of a PLS-DA analysis of a data matrix containing two groups: controls at 24 hours and the D-serine 500 mg/kg group at 24 hours post-dose. The results of the PLS-DA ranking analysis are not shown here, but Solutions Labs illustrated that some of the variables in Table 6 may be potential biomarkers. The trend plots of the ion intensities of 10 variable metabolites from Table 6 across the four control and the five 500 mg/kg rats are shown in Figures 6 and 7.

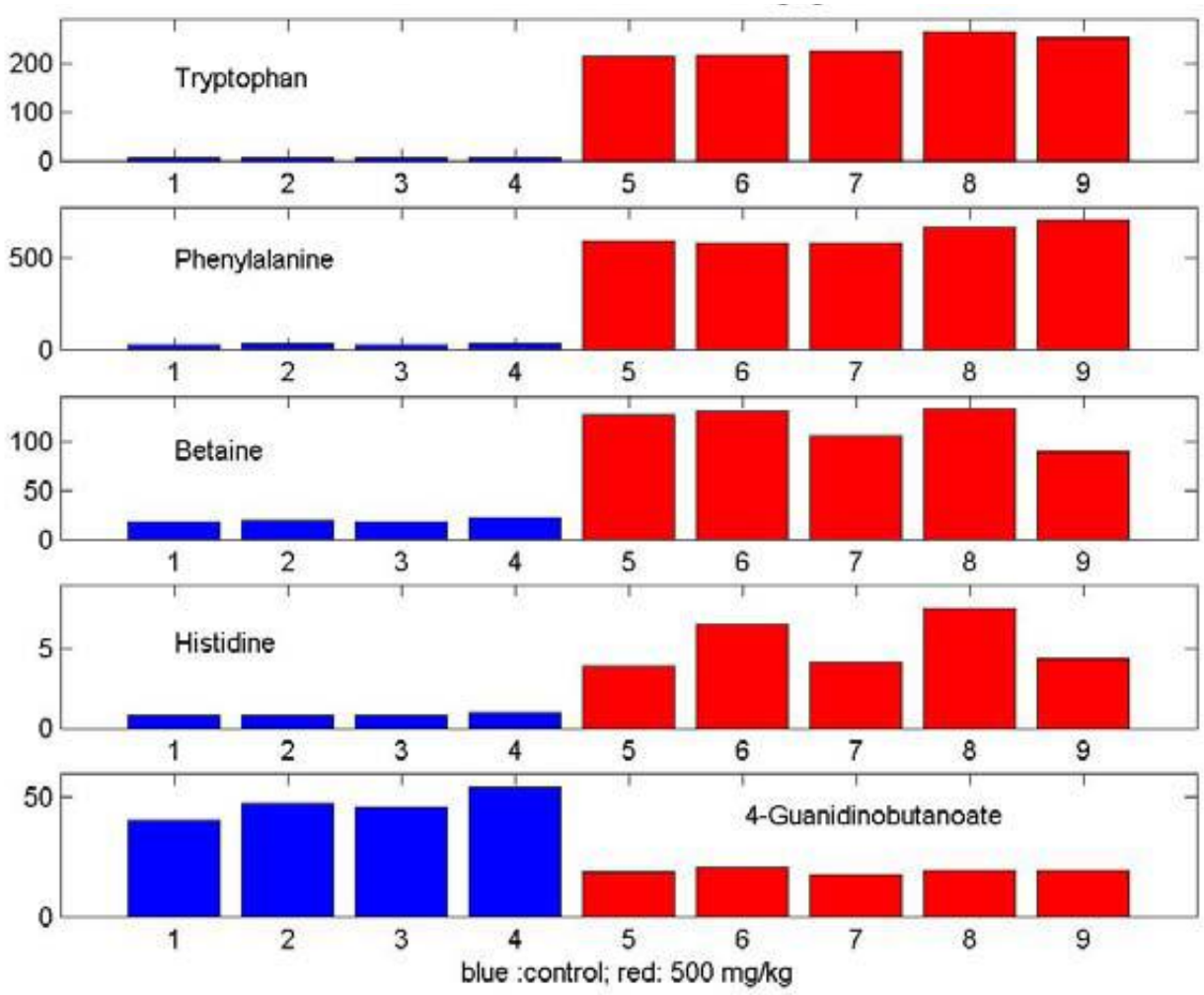


Figure 6: LC/MS normalized ion intensities across urine samples for tentatively identified metabolites: Tryptophan, Phenylalanine, Betaine, Histidine, and 4-Guanidinobutanoate

Animals 1 to 4 are controls at 24 hours post-dose and animals 5 to 9 are the 500 mg/kg D-serine dosed group at 24 hours post-dose.

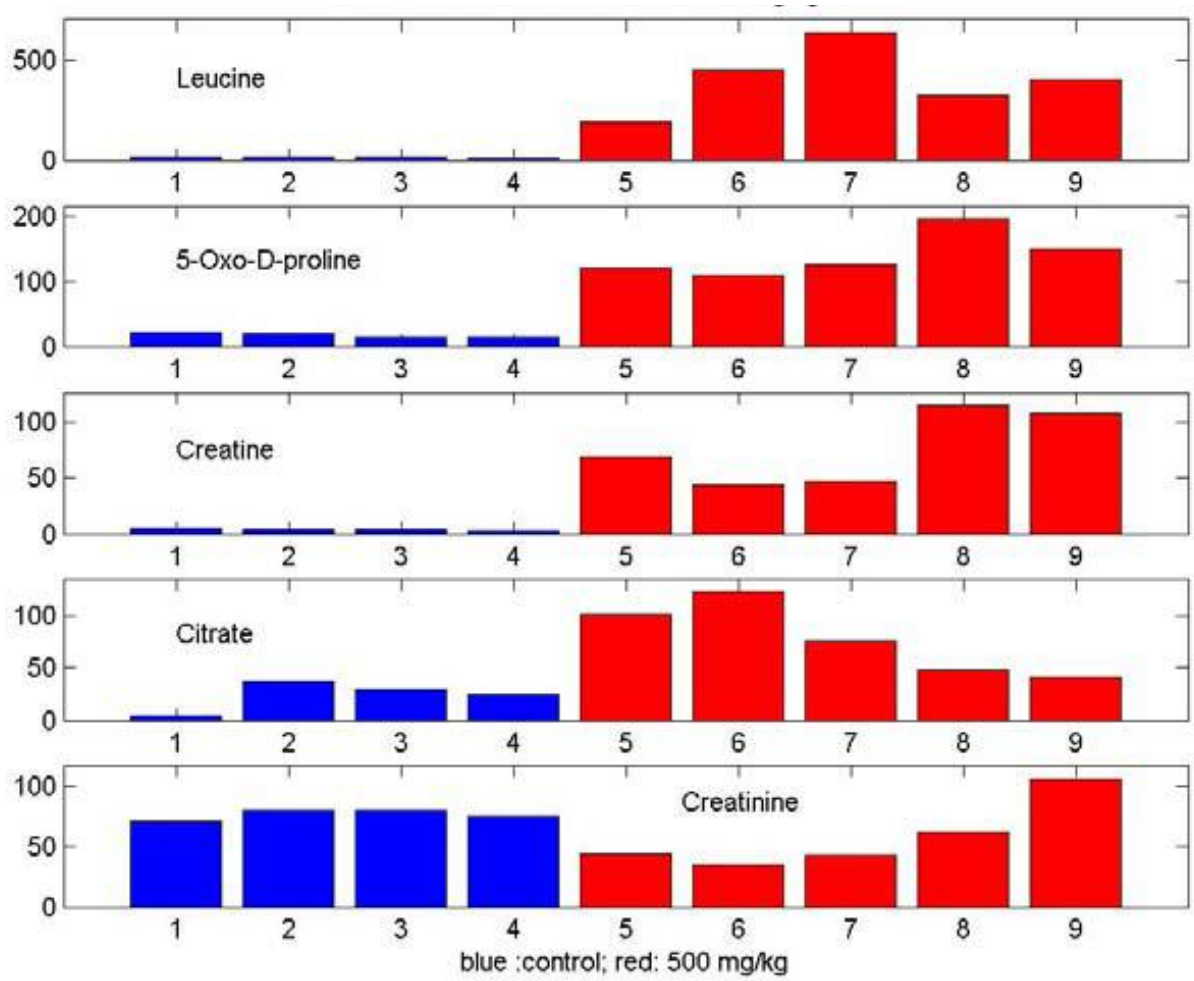


Figure 7: LC/MS normalized ion intensities across urine samples for tentatively identified metabolites: Leucine, -Oxo-D-Proline, Creatine, Citrate, and Creatinine

Animals 1 to 4 are controls at 24 hours post-dose and animals 5 to 9 are the 500 mg/kg D-serine dosed group at 24 hours post-dose.

An absolutely definitive identification of variable metabolites would require fragmentation measurements using LC-MS/MS, but it is possible that an effort to expand the pure metabolite retention time library to generate more candidates may be more useful. Expansion of such a database would lead directly to the discovery of many potentially important biomarkers.

3.3. Puromycin (PUR) NMR

3.3.1. NMR Spectroscopy of Urine Samples

The urinary assessment and analysis of the kidney toxin PUR using NMR was similar to that described above for D-serine, but different data pre-processing steps were necessary due to the nature of the samples. As previously stated above for D-serine, the highest dose inducing metabonomic perturbations in kidney function was first determined. Initial NMR analysis indicated that the highest PUR dose group (150 mg/kg) induced perturbations in the profile of urinary metabolites (Figure 8). The representative NMR spectra shown in Figure 8 show the changes in endogenous components of urinary metabolites observed 120 h post-exposure in rats given a single i.p. dose of normal saline (Figure 8A) and 150 mg/kg PUR (Figure 8B), respectively. Puromycin was selected as a model toxin for the induction of glomerular damage. However, the toxic effects of PUR on the glomerulus do not occur until approximately five days post-exposure while other toxic effects were evident 24 hours post-exposure (i.e. elevated BUN and decreased total protein; see Table 5, Volume 1). Thus, it appears that there are two mechanisms of toxicity occurring following exposure to PUR. The assessment of PUR induced toxicity in Sprague Dawley rats has previously been evaluated using NMR (Holmes and Shocker, 2002) and indicated significant increases in urinary levels of acetate, alanine, creatine, formate, glucose taurine and trimethylamine-N-oxide metabolites, with corresponding significant decreases in levels of citrate and 2-oxoglutarate metabolites.

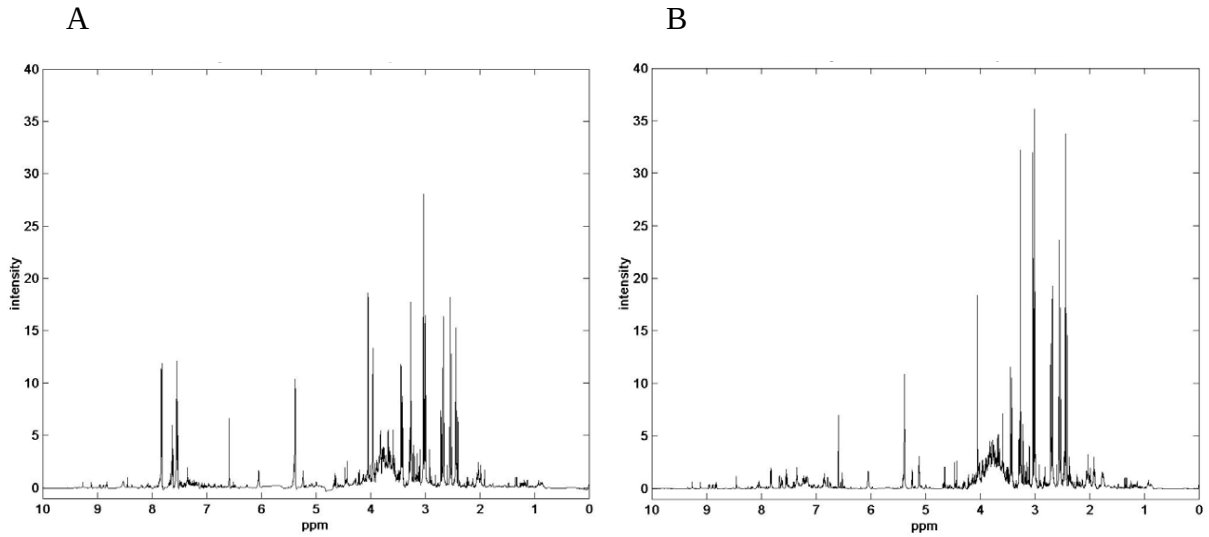


Figure 8: Representative 600 MHz ^{1}H NMR rat urine spectra from dosed rats

A) Normal saline control and B) 150 mg/kg PUR at 120 h post-dose.

Peaks have been scaled using area of TSP internal standard. Water and urea peaks have been removed and spectrum is sum intensity normalized.

3.3.2. NMR PCA Analysis

After manual phasing and baseline correction, the data was binned with 0.04 ppm bins between 0 – 10 ppm and exported after excluding water and urea resonance regions (4.7 - 4.825 and 5.65 – 5.95 ppm, respectively). The initial data matrix will consist of controls (n = 4) and 150 mg/kg (n = 4) at 120 hours. NMR spectra were normalized by sum intensity normalization, and scaling was performed using Pareto scaling. The percent variation by PCA component revealed a significant concentration of the variance within the first two components (Figure 9). Moreover, the associated scores plot shows that the control and PUR exposed treatment groups cluster coherently and that the first component corresponds to toxicity related perturbations (Figure 10).

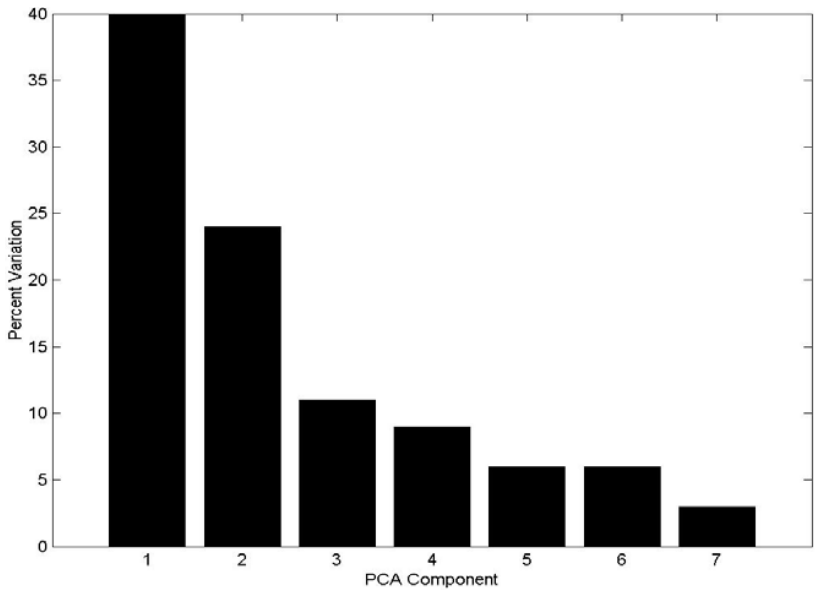


Figure 9: Percent variation of NMR data for rat urine analysis by PCA component of 120 hour saline controls versus 150 mg/kg PUR binned spectral data

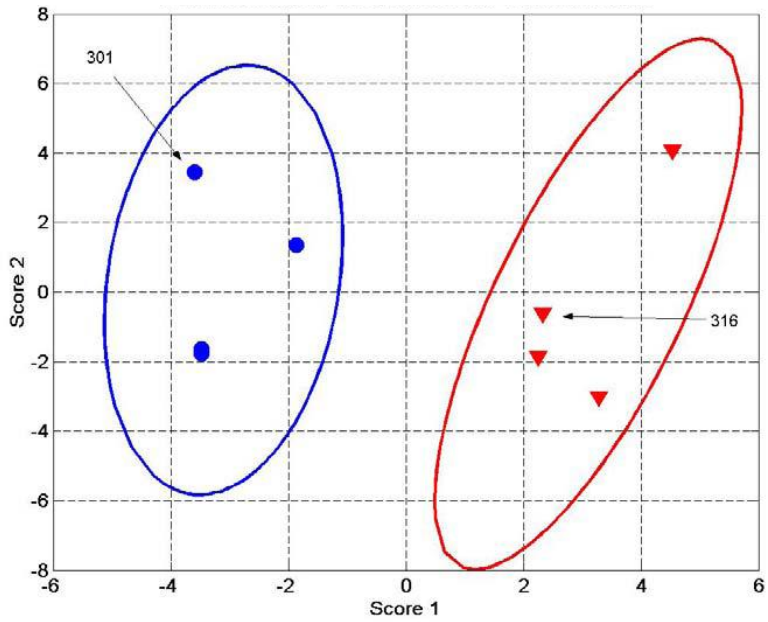


Figure 10: Scores 1 and 2 of PCA analysis of 120 hour PUR experiment

Saline controls (blue) and 150 mg/kg puromycin (red). Two animal samples are pointed out for reference.

3.3.3. Chenomx Puromycin (PUR) Profiling

During the data acquisition process for the PUR study, there were indications of the presence of proteins in the urine samples (i.e. broad peaks in NMR spectrum). Urinary proteins can interfere with efforts to acquire the cleanest small molecule profiling that is desired. It is not entirely clear how this impacted the NMR data analysis, but protein was indicated in urine samples at day-5 following exposure to the highest PUR dose (150 mg/kg). Using the automatic processing (i.e. phasing and baseline correction) of the Chenomx processor on these samples leads to spectra on which one cannot apply the additional reference de-convolution that is recommended by Chenomx. The processing package documentation indicated that such situations occur when the spectra have insufficiently good phasing and baseline correction. Thus, a great deal of effort was put into manual phasing and cubic polynomial baseline correction (particularly on the baseline correction). The structural improvements of the spectra were dramatic, and this fact suggested that the prior studies should also be repeated with optimized processing. Initial analysis was restricted to samples for which there was also LC-MS data and

multivariate factorization scores and loadings studies, with detailed profiling of individual samples, was used for the purposes of candidate biomarker identification.

The excellent group separation for the PCA analysis implies that the supervised PLS-DA method will give an accurate indication of the contributions of variables to group separation through a loading analysis in the first component. This analysis will follow after the profiling of two samples (data not shown). Although there are noticeable differences, care must be taken when trying to impose observations of just two samples onto conclusions concerning the entire data set, because there exists a considerable amount of variation between samples within a group. For example, examination of two other samples (a control and 150 mg/kg PUR) indicated that while a second urine sample from a PUR exposed animal was quite similar to its cohort sample indexed below (Table 7), there were some marked differences. Therefore, although the two samples below were annotated (Figure 11) two other samples (a control and PUR treated) were also used during the PLS-DA annotation to avoid drawing incorrect conclusions regarding the entire data set from the first two- sample comparison study. The Chenomx profiling of the first set of samples identified interesting regions (i.e. potentially differentially regulated metabolites) of the spectra and is presented in Table 7.

Table 7: Preliminary identification and concentration estimates of the un-normalized data from rat urine of pre and post (120 hour) dosing (150 mg/kg PUR)

The entries of "NA" indicate that these compounds have not yet been fit (quantitated).

Index	Metabolite	0 h Predose (mM)	120 h PUR (150mg/kg) (mM)
1	1-Methylnicotinamide	0.439	0.439
2	2-Aminoadipate	NA	0.558
3	2-Aminobutyrate	0.172	NA
4	2-Hydroxyisobutyrate	0.052	0.090
5	2-Oxoglutarate	6.211	17.634
6	4-Hydroxyphenylacetate	NA	0.571
7	Acetate	0.245	0.539
8	Alanine	0.133	0.201
9	Allantoin	11.364	12.208

10	Asparagine	NA	1.096
11	Betaine	0.312	NA
12	Carnosine	NA	0.542
13	Citrate	8.067	14.284
14	Creatine	0.479	0.727
15	Creatinine	3.492	4.880
16	Dimethylamine	0.641	1.158
17	Formate	0.534	0.935
18	Fumarate	0.118	0.423
19	Glucose	2.885	4.063
20	Glutamine	NA	1.550
21	Hippurate	5.458	1.128
22	Histidine	NA	0.501
23	Kynurenine	0.201	0.224
24	Lactate	0.238	0.296
25	Methylamine	NA	0.456
26	N,N-Dimethylglycine	0.223	0.175
27	N-Acetylglutamate	NA	0.398
28	Ornithine	NA	1.041
29	Phenylacetylglycine	0.406	0.701
30	Pyroglutamate	NA	1.732
31	Succinate	0.598	1.603
32	Sucrose	1.479	1.399
33	Taurine	2.871	5.220
34	Trigonelline	0.422	0.677
35	Trimethylamine N-oxide	0.458	1.235
36	Tryptophan	NA	0.117
37	Uracil	1.684	NA
38	Urea	47.955	59.655
39	Valine	NA	0.048
40	Xylose	0.405	NA
41	trans-Aconitate	2.226	2.394

The identification indices of the metabolites in the above table were used to annotate the spectra divided into 2 ppm intervals (Figure 11).

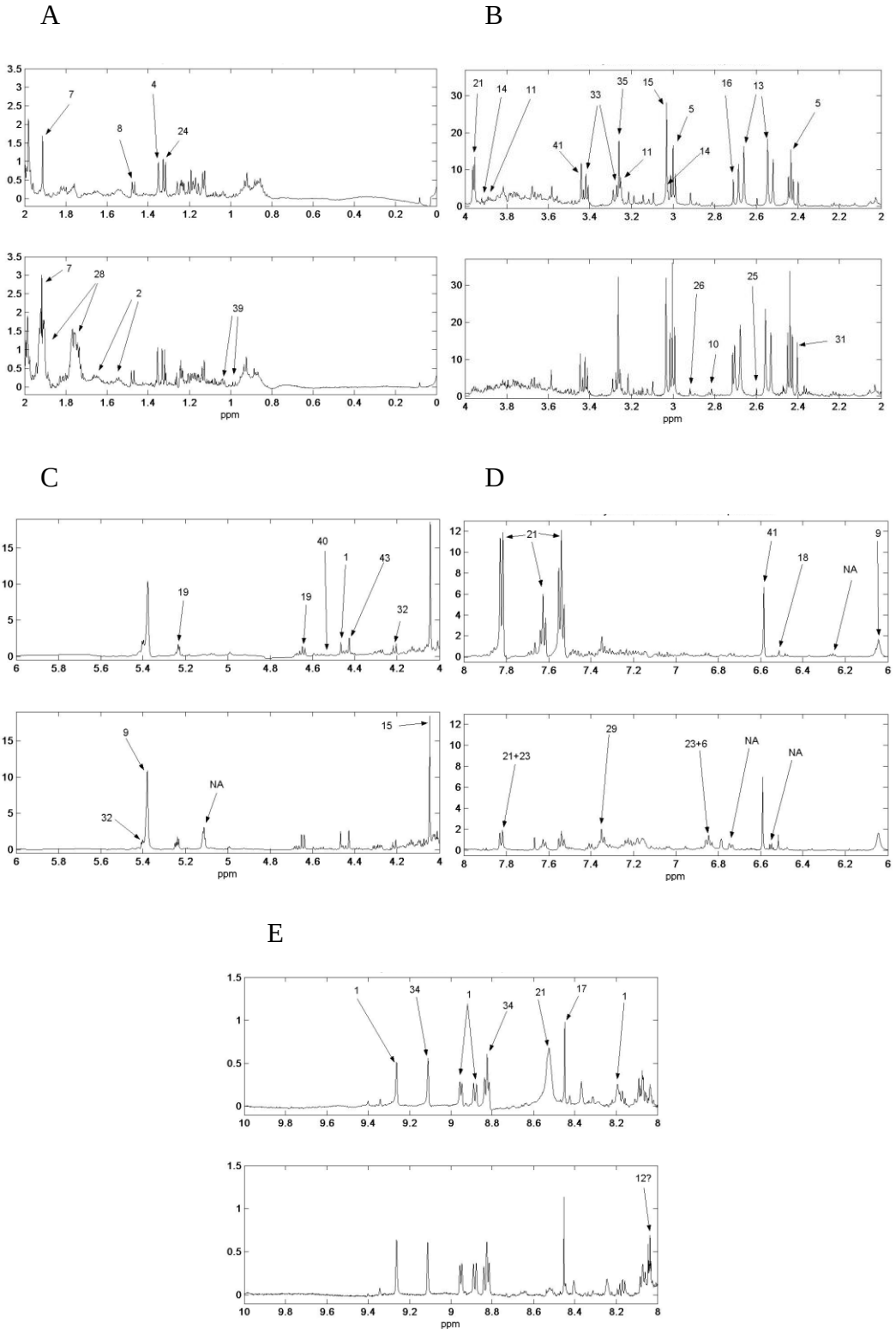


Figure 11: Representative annotated NMR traces

(A – E) of predose (top pannel) and 120 hours PUR post-dose (bottom pannel) rat urine. The traces of the two samples are in megahertz frequency intervals of 2ppm. A, 0 – 2 ppm; B, 2 – 4 ppm; C, 4 – 6 ppm; D, 6 – 8 ppm and D, 8 – 10 ppm. The number index labels of the figure correspond to metabolite numbers identified in Table 7. Signal was scaled using area of internal standard peak. Designation of "NA" means not in library. Two labels combined with '+' indicate overlapped signatures.

The above spectra are instructive, but some of the results are surprising, e.g. regarding alanine and 2-OG that did not generalize to a comparison that was performed with the second set of samples. Therefore, these might be artifacts of the particular sample set chosen. Despite this, it is possible to begin to understand the PLS-DA loading analysis in terms of the identity of relevant variables. However, the 0.04 ppm binned analysis, as with other toxicants, also suffers from a few cases where individual bins share signals from spectrally adjacent metabolites thus compromising their value as discriminators. The PCA analysis strongly suggested that the PLS-DA first component loadings would reveal variable influence on the group separation. The actual PLS-DA X-block/Y-block variation and scores analysis (data not shown) confirms this conjecture.

3.3.4. Preliminary Investigation of Candidate NMR Biomarkers of PUR Exposure

As in previous PLS-DA regression analysis, four stratifications were chosen for critical p-values: p less than 0.00033, p between 0.00033 and 0.026, p between 0.026 and 0.05, and p greater than 0.05. The first p interval corresponds to the Bonferroni-Holm correction, and the second interval corresponds to a False Discovery Rate correction (Q = 0.2). Only metabolites with "p" values ≥ 0.05 were considered significantly altered (Table 7). This analysis allowed us to present a number of potential biomarkers for comparison to those that were published in the above-cited references.

Although there were similarities in the differentially regulated metabolites in the urine of rats exposed to PUR when compared with that previously reported (Holmes and Shocker, 2002), differences were noted (i.e. taurine not elevate, creatine not elevated, TMAO not elevated, 2-

oxoglutarate not decreased). Interestingly, metabolites such as taurine and TMAO, which were not listed in Table 7 due to their large p-values, appeared to be regulated when concentrations were measured using Chenomx profiling software. This discrepancy might be an artifact of the small sample size and the sensitivity of the t-test to detect outliers. Furthermore, metabolites identified in the literature were obtained from a different strain of rat than was used in the present study. This issue will need to be investigated in follow-up studies in order to resolve this problem.

Another interesting feature of the potential biomarker list is the up-regulation of both 1-methylnicotinamide and trigonelline which is in contrast with the role that these metabolites play in the other kidney toxin experiments. These results must be verified to determine that they are reliable and not just an artifact of the normalization process.

Future analysis of the 48 hour PUR samples will be interesting because it has been implied in the literature (Shockcor and Holmes, 2002) that kidney proximal tubule damage might be present. Therefore, any potential biomarkers appearing at 48 hours might be comparable to those biomarkers identified in the D-Serine study.

Table 8: Differentially regulated metabolites in urine of rats exposed to PUR (150 mg/kg)

Metabolite concentrations considered significantly altered if PLS-DA "p" values ≤ 0.05 when compared to controls (see Table 7).

Up-Regulated	Down-Regulated
4-Hydroxyphenylacetate	2-Aminoadipate
1-Methylnicotinamide	Citrate
Acetate	Hippurate
N-Acetylglutamate	Kynurenine
Asparagine	Valine
Dimethylamine	Xylose
Glucose	
Glutamine	
Histidine	
Methylamine	
Ornithine	

Pyroglutamate	
Sucrose	
Trigonelline	

3.4. Puromycin (PUR) LC/MS

3.4.1. LC/MS Spectroscopy of Urine Samples

Acquisition of the LC-MS positive ionization data was obtained from the Laboratory of Translational Medicine at Harvard Medical School as previously described above for D-serine metabonomics. The preprocessed data (Waters MarkerLynx output) was transferred to Solutions Labs for preliminary analysis. Data acquisition and processing using MarkerLynx was performed as described above for D-serine. LC/MS data was acquired on rat urine from the highest dose (150 mg/kg) of PUR to induce significant effects on the kidney as determined by histopathology and clinical chemistry findings. The results and conclusions drawn were similar as for the previous D-serine study. However, the ratio between the variance in the first and second PCA component was smaller. This fact is reflected in the scores plot in which both components now play a role in separating the PUR dosed groups from control. Comparison of the PCA scores of the dosed group and that of the control again indicated strong clustering. The time of maximum perturbation is on or after day 5 which is consistent with results reported in the literature (Holmes et al., 1998).

3.4.2. LC/MS PCA Analysis

After variable filtering and data transformation as previously described above, the data was subjected to PCA analysis. PCA analysis was first used to determine the effects of PUR exposure on rats with respect to dose, and indicated that the only dose of PUR to induce any significant effects was the highest dose used (150 mg/kg; data not shown). To assess the degree to which the toxic response altered the metabolome of exposed animals, the time-dependent metabolic profiles of the highest dosed PUR group (150 mg/kg) was investigated using PCA analysis of data matrices which included only the control subjects and the high dose subjects (Figure 12).

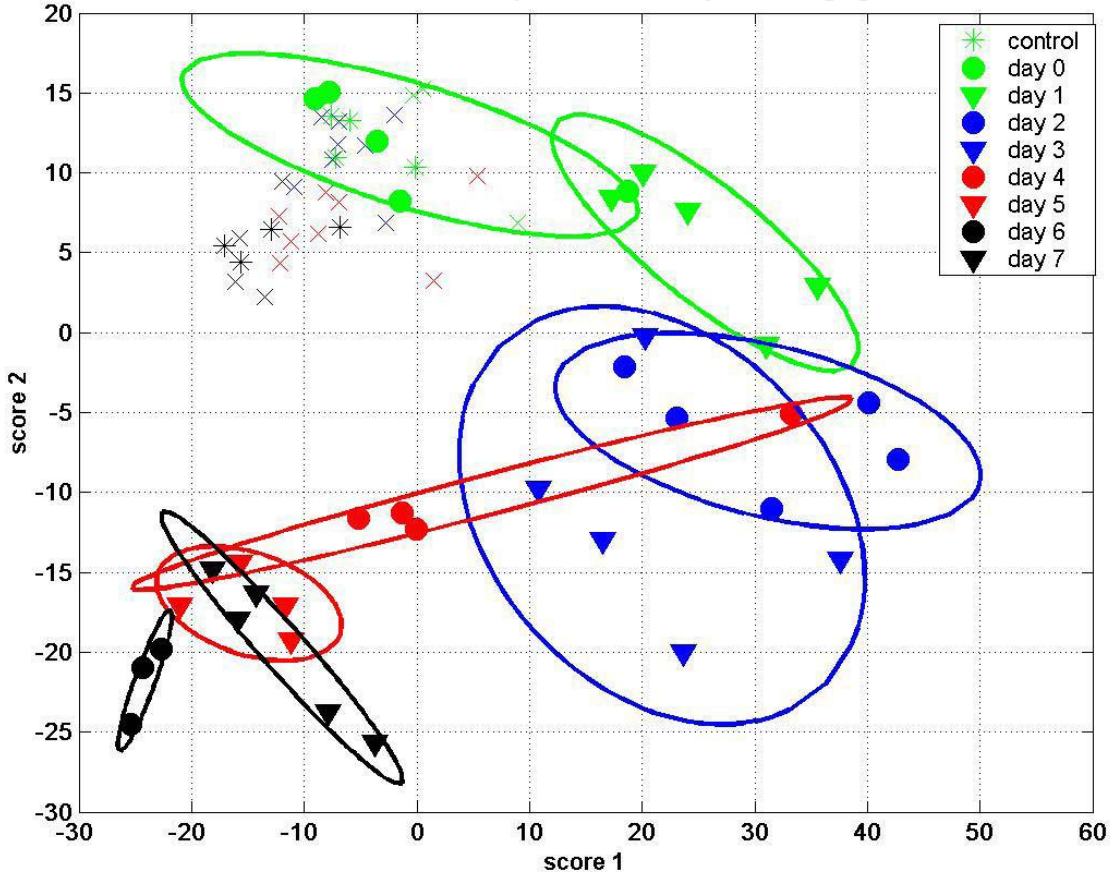


Figure 12: Scores plot for the first two PCA components of the PUR

The points plotted with stars (*) and crosses (x) are the control group whose color-coding matches that of the dosed group. The control points alternate in time between stars (even days) and crosses (odd days). Post-dosed 150 mg/kg animals clearly cluster away from their pre-dosed state and away from controls.

3.4.3. Preliminary Investigation of Candidate LC/MS Biomarkers of PUR Exposure

Initially, a comparison between controls and the 150 mg/kg PUR-treated group at a post-exposure time of 120 hours was investigated because published toxicological research indicated that overt glomerular damage occurred at this time. However, a clear separation between PUR-treatment and control groups is evident much earlier, i.e. at 48 hours post-exposure, and this separation indicated that the possibility that another mechanism of toxicity was present. In fact, some publications listed tubular damage as another consequence of PUR exposure (Shockcor and

Holmes, 2002). Therefore, an analysis of the PUR data at 48 hours was initiated to explore whether the differential expression of candidate biomarkers might be similar to some of the biomarker profiles tentatively identified in the D-serine study. Any similarities in potential biomarkers would be relevant, in part, to the overall project goal of establishing metabolite patterns to classify toxicity mechanisms.

The LC/MS data was preprocessed as previously described above, and multivariate (PCA and PLS-DA) statistics was applied to a data matrix consisting of the controls and 150 mg/kg PUR group at 48 hours post-dose. After verifying that the two treatment groups were well separated, an attempt to identify variables using Solutions Labs retention time library was applied to all variables that were within a 25 ppm window of their KEGG-based metabolite mass database. Candidate biomarker metabolites for possible positive identification based on close retention times were tabulated and presented in Table 9.

Table 9: Potential biomarker candidates tentatively identified using LC/MS data and KEGG database

Results of applying automated mass identification along with retention time library data to make tentative identifications of variables in the PUR data set that was filtered to include only variables expressed consistently across pre-dose animals.

Metabolite	RT Library	RT Measured	PLS Rank (log)	PLS Rank
Leucine	1.98	1.912	341	53
Thiamin	0.67	0.675	411	58
Glutamate	0.68	0.842	46	89
Valine	0.99	0.937	133	208
Creatine	0.74	0.689	3	340
Maltose	0.72	0.842	410	571
Betaine	0.67	0.650	395	594
Phenylalanine	2.5	2.470	494	715
5-Oxoproline	1.56	1.388	1333	858
5-Hydroxytryptophan	2.15	2.186	955	933
4-Guanidinobutanoate	1.11	0.960	1240	1121
Citrate	1.2	1.166	1291	1130
Adenine	0.87	0.910	989	1263

Tryptophan	3.11	3.105	944	1377

The above table also includes the PLS-DA rankings of the candidate metabolite identifications for PLS-DA analyses that were conducted with and without logarithmic transformation of the data prior to unit-variance scaling of the variables. There were a total of 1378 variables in this data set. Since creatinine does not appear in the above table, Solutions Labs investigated the cause of this omission, and determined that it was indeed present in the data, but it was about 30 ppm removed from its exact mass due to saturation effects. The sum-normalized expression levels of metabolites listed in Table 9 above across control and PUR treatment groups, including creatinine, is illustrated in Figures 13-15.

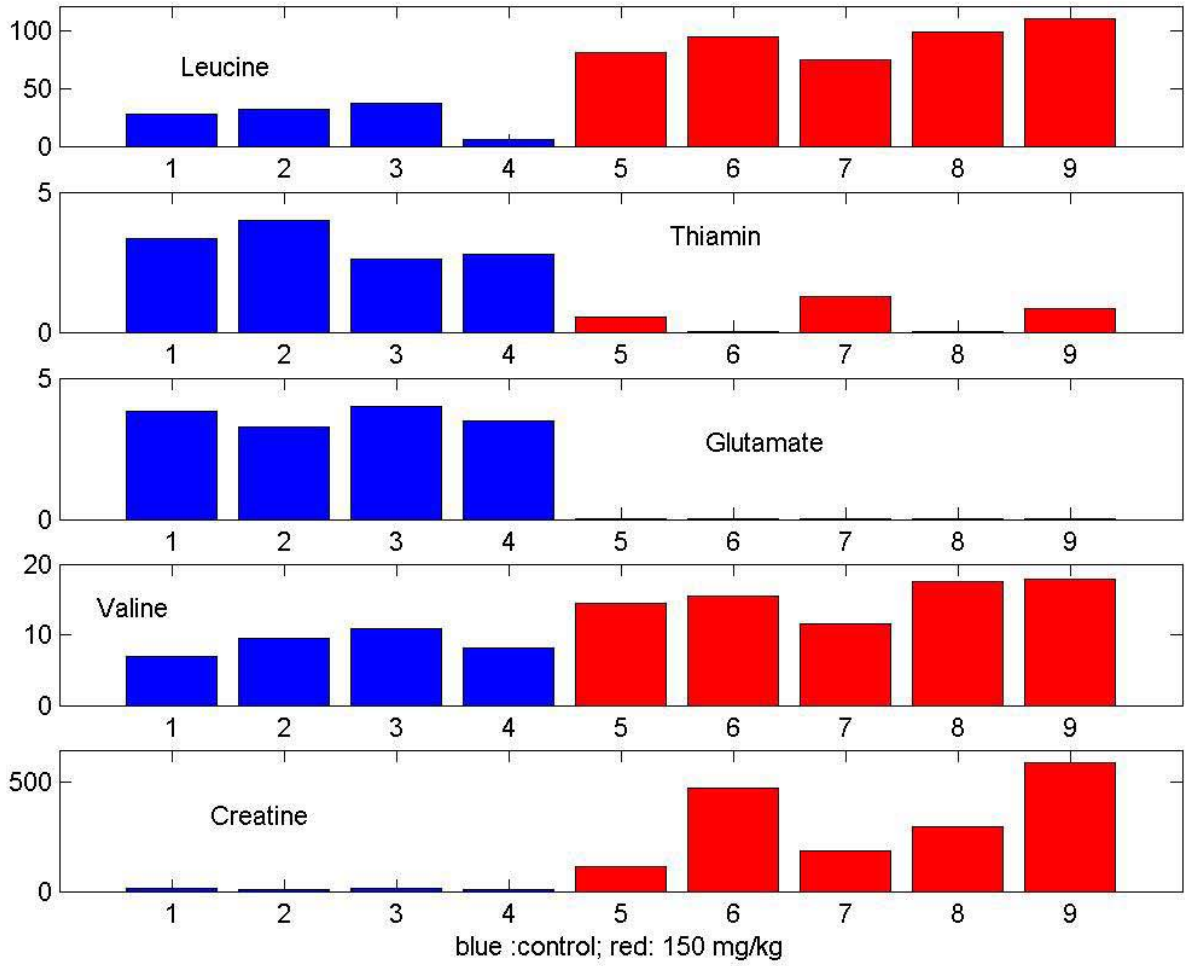


Figure 13: PUR (150 mg/kg) and controls sum-intensity normalized LC-MS positive mode data (48 hours post-dose) for the select variable metabolites leucine, thiamine glutamate and creatine indicated in intensity bar graphs

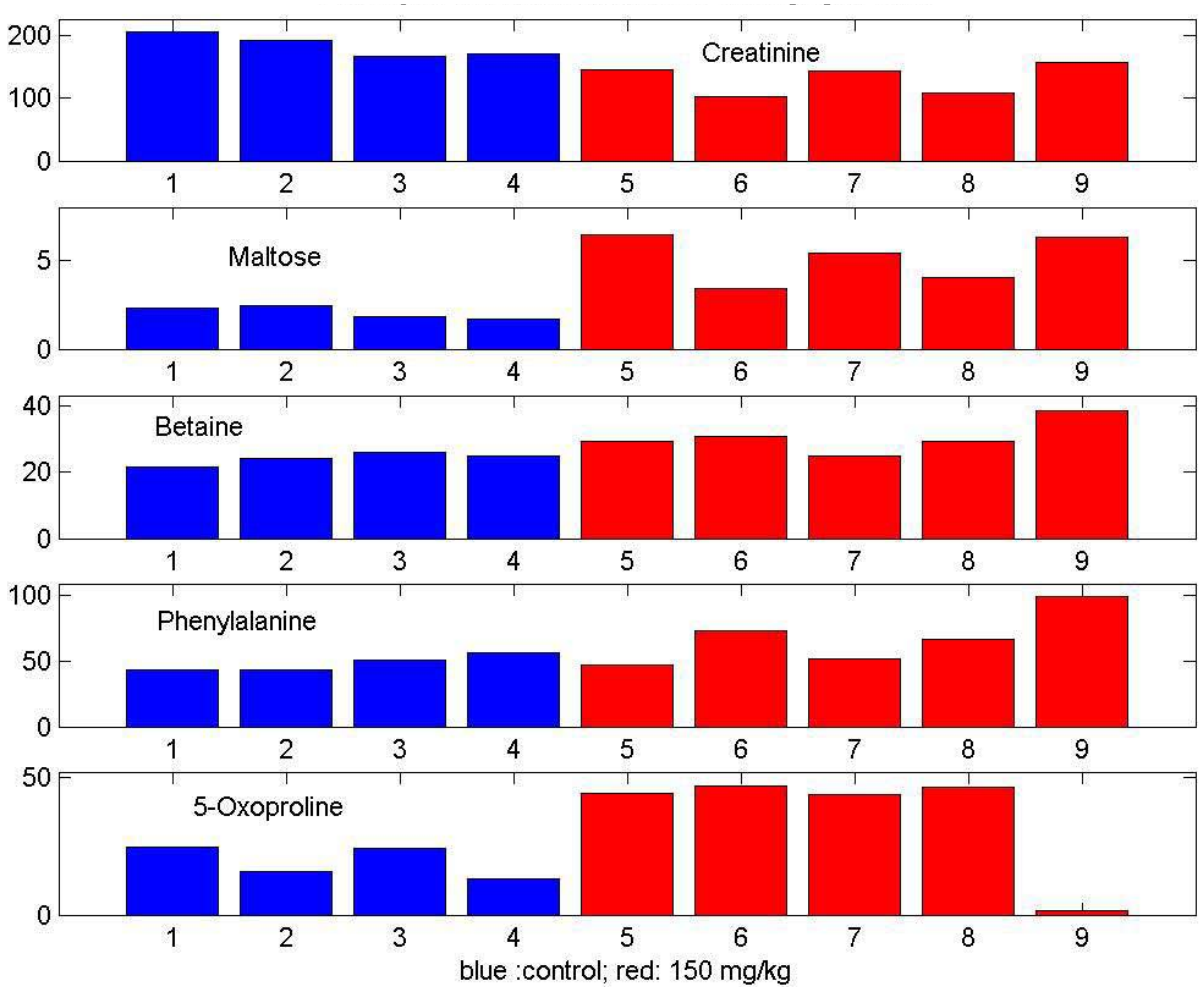


Figure 14: PUR (150 mg/kg) and controls sum-intensity normalized LC-MS positive mode data (48 hours post-dose) for the select variable metabolites creatinine, maltose, betaine, phenylalanine and 5-oxoproline indicated in intensity bar graphs

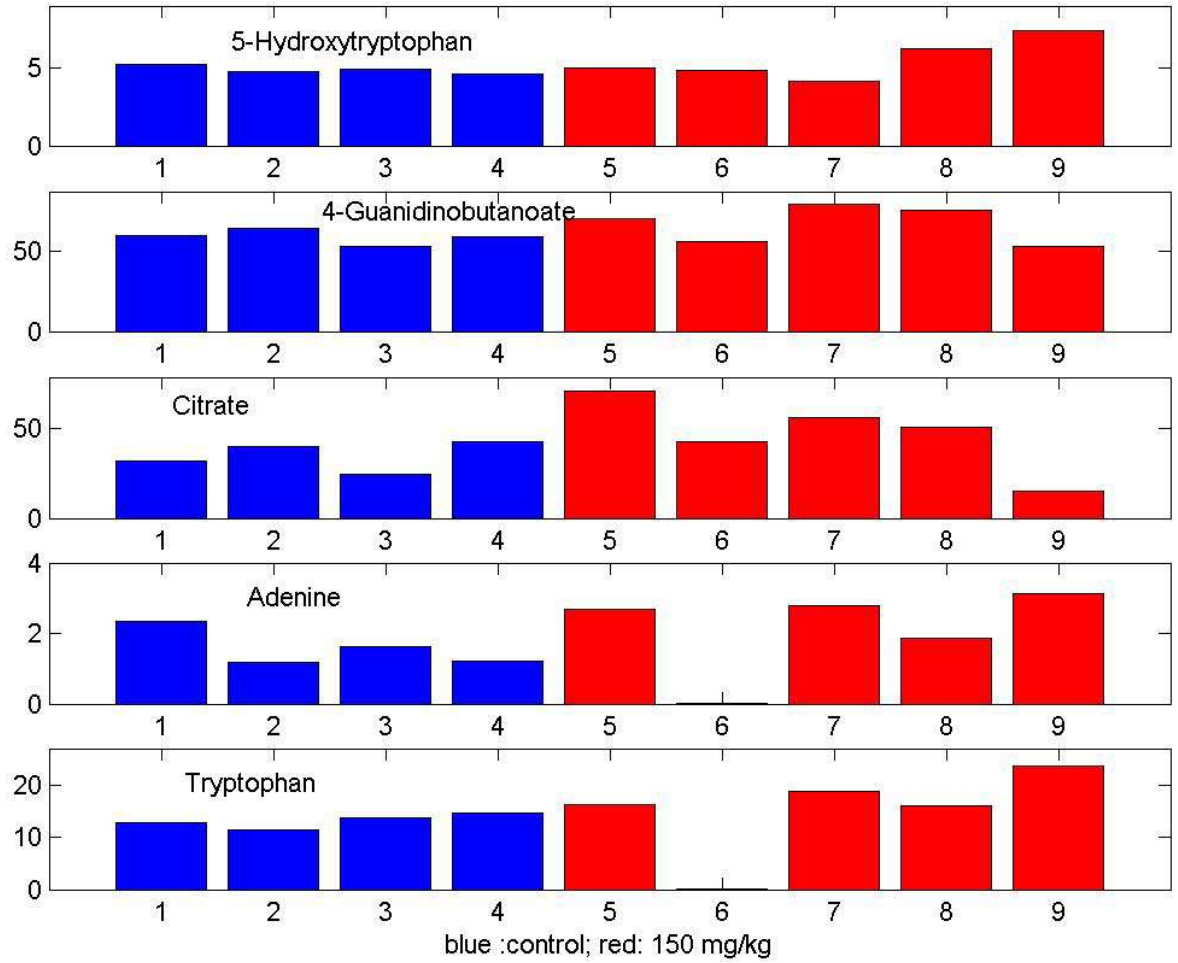


Figure 15: PUR (150 mg/kg) and controls sum-intensity normalized LC-MS positive mode data (48 hours post-dose) for the select variable metabolites 5-hydroxytryptophan, 4-guanidoinobutanoate, citrate, adenine and tryptophan indicated in intensity bar graphs

One noteworthy observation concerns the unexpected expression characteristics of the citrate candidate metabolite. Citrate also presented problems in previous analysis of the D-Serine experiment, and development of a possible explanation of its behavior within the positive mode LC-MS data acquisition is still in progress. Otherwise, there were no obvious inconsistencies between the LC-MS data and the NMR data for the above compounds. Moreover, the LC-MS data appears to confirm that creatine is a strong candidate as a biomarker for the effect of PUR exposure at 48 hours post-dose. Due to the limited scope of the metabolite retention time database, definite identifications within the LC-MS data of marker candidates extracted from the

NMR data could not be made, but by using compound mass, several compounds within the LC-MS data that might support the NMR analyses were identified (Table 10).

Table 10: Potential biomarker candidates of PUR exposure tentatively identified from LC/MS data using compound mass

Potential Metabolite	Possible RT (min)
Hippurate	3.93
Phenylacetylglycine	4.51
Trigonelline	0.64
1-Methylnicotinamide	0.64
Trans-Aconitate	1.59

The sum-normalized expression levels of metabolites listed in Table 10 above across control and PUR treatment groups is illustrated in Figure 16. All of the potential metabolites identified in Table 10 are within 30 ppm of their exact masses, and it was encouraging that the candidate metabolites hippurate and phenylacetylglycine exhibit the same strong differential regulation predicted by the results of the NMR analysis. It will be important is recommended that retention times of these compounds be acquired as soon as possible in order to confirm their identity. The other three candidates also appear consistent with the NMR data.

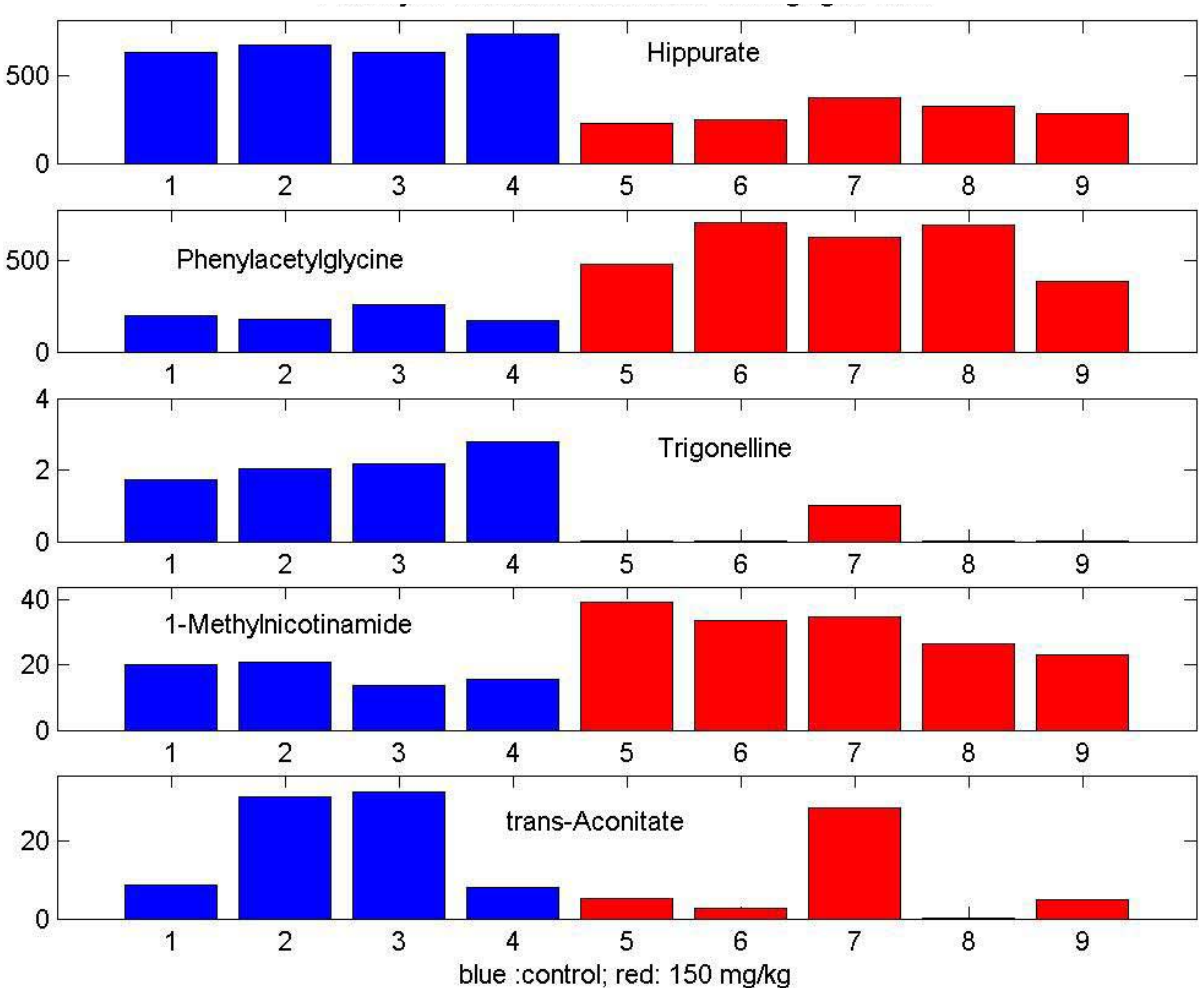


Figure 16: Intensity bar graphs of PUR (150 mg/kg) and controls sum-intensity normalized LC-MS positive mode data (48 hours post-dose) for the select variable metabolites hippurate, phenylacetylglycine, trigonelline, 1-methylnicotinamide, and trans-aconitate based on mass

A search for the metabolites taurine, succinate, and 2-oxoglutarate was performed and indicated that taurine and succinate appeared to be entirely absent. It is possible that taurine and succinate have strong signatures in the negative ionization mode rather than the positive mode. The 2-oxoglutarate candidate metabolite does not show differential regulation. It is also possible that the candidate variable is not 2-oxoglutarate or perhaps 2-oxoglutarate behaves in the same anomalous fashion as citrate. Again, it is suggested that retention times be acquired for these metabolites in order to confirm their identities or uncover potential technical problems.

An analysis of the PUR 48 hour post-dose data, for both NMR and LC-MS, was initiated in order to characterize a potential second toxicity mechanism induced by PUR and to draw a possible connection with the D-serine expression data with respect to establishing common biomarkers of tubular damage. In the LC-MS data, it is found that the PUR 48 hour post-dose data does not show the same strong up-regulation of biomarker metabolites such as tryptophan, betaine, and phenylalanine. However, creatine, leucine, 5-oxoproline and valine are common up-regulated biomarkers. Therefore, it is possible that there is a common toxicity mechanism for kidney tubular damage, and the differences were due to dosage-dependent effects.

Comparisons of the NMR data should be made with caution; the strong up-regulation of phenylacetylglycine in the PUR study data is absent in the D-Serine study urine profiles. However, nearby phenylalanine resonances (around 7.4 ppm) were observed in the D-serine study samples. Thus, some overlaps may mask the relative concentrations of these compounds. Additional work will be needed to characterize and resolve these ambiguities.

3.5. Hippuric Acid and Amphotericin B

Histopathology and clinical chemistry results indicated that no effects of hippuric acid or AmpB could be observed following terminal sacrifice of the animals. Therefore, no NMR or LC/MS analysis was performed on any of the rat urine samples obtained from these studies.

3.6. Kidney Toxicant Pair-Wise Analysis and Variable Selection

As an initial proof-of-concept investigation, Solutions Labs implemented a simple t-test based variable selection process to identify a few variables that might indicate the existence of a "panel of biomarkers" for identification and distinguishing toxic kidney responses. At this stage, they used a t-test method without correcting for multiple hypotheses testing in order to select some variables that might satisfy their needs. The results of this t-test method using the LC/MS data are presented in Figure 17.

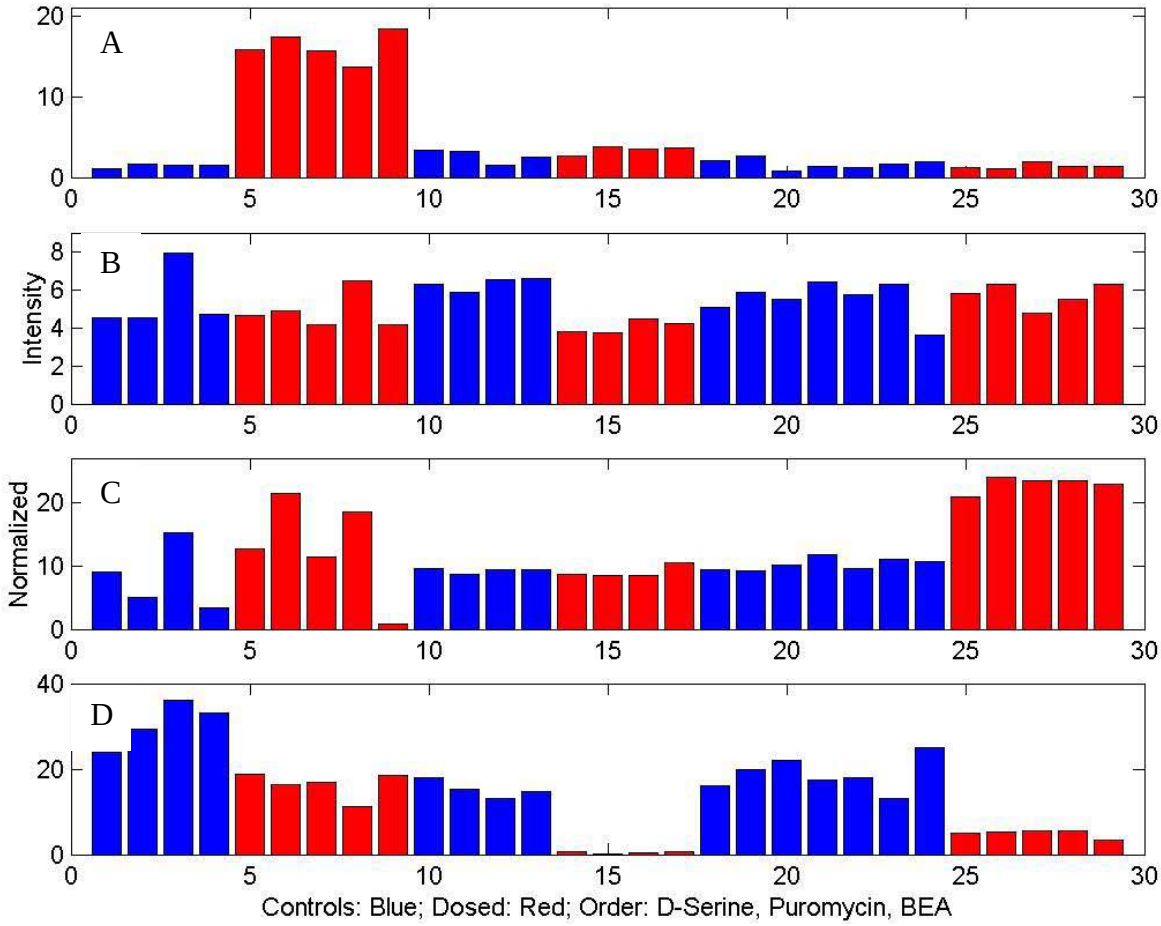


Figure 17: Normalized intensity profiles across all kidney toxicant samples for variables *A), m/Z=150.0589 (RT = 1.11); B), m/Z=221.0927 (RT=3.98); C), m/Z=228.1949 (RT=7.86); and D), m/Z=338.0875 (RT=3.98). Controls are blue and dosed groups are red and presented in the order from left to right as D-serine, PUR, and Bromoethylamine*

The above cross-sample profiles show candidate biomarkers that might be used to distinguish between different kidney toxicants such as D-serine, PUR and bromethylamine (BEA), or kidney damage in general. For example, the first profile (Panel A; Figure 17) shows strong up-regulation for an unknown metabolite with a m/Z ratio of 150.06 in the D-serine experiment and little changes for this metabolite with the other kidney toxicants. The second and third profiles (Panels B and C; Figure 17) suggest selective metabolite biomarkers for PUR (m/Z ratio of 221.09; down-regulated) and BEA (m/Z ratio of 228.19; up-regulated). The last panel (Panel D) shows a biomarker metabolite (m/Z ratio of 338.09) that is down-regulated for all three

kidney toxins. If it can be confirmed that this last metabolite biomarker candidate is consistently down-regulated across all classes of kidney toxicants, then it could be used as a good predictive biomarker specific to nephrotoxicity. Identifying, cataloging, and biologically interpreting all variables with properties similar to the ones illustrated in Figure 17 will require a great deal of effort. However, these preliminary results support the plausibility of the current strategies for development of an "Expert Software System" for analysis of biomarkers as an early indicator of potential target-organ toxicity. By complementing NMR measurements with more sensitive LC-MS profiles, it will be possible to greatly extend the scope of urinary metabolic profiling. However, this is contingent on the establishment of an LC-MS metabolite library and metabolite identification procedure.

4. CONCLUSIONS

In this study we examined the urinary metabolite profiles from rats following a single exposure to the kidney toxicants D-serine, puromycin, hippuric acid and amphotericin B at various doses, and as a function of time post-dose. In toxicology, such dose-time metabonomics studies are important for an accurate determination of the severity of biological effects, and for biomarker identification that may be associated with toxicity. The metabonomics analysis yielded a dose-response curve in principal component analysis space, and was able to detect exposure to D-serine and puromycin at much lower doses than standard clinical chemistry measures. Additionally, characteristic features in the urinary metabolite profiles could be ascertained as a function of dose.

The results showed common features and some unique features in urinary metabolite profiles when analyzed by NMR and LC-MS, respectively. For example, with animals exposed to D-serine, the urinary metabolites histidine, phenylalanine, proline, leucine and betaine were observed to be elevated, while the metabolite creatinine was observed to be decreased, when measured by NMR and LC/MS, respectively. Generally, a higher amount of metabolites were identified using NMR than LC/MS. Although LC/MS analysis is more sensitive than NMR, a much more extensive database exists for NMR data. The metabolites identified in urine of rats exposed to D-serine using NMR and LC/MS correspond to the nephrotoxicity of D-serine previously reported (Carone and Ganote, 1975). Previous work has shown that D-serine induced kidney tubule necrosis was accompanied by aminoaciduria, glucosuria and proteinuria (Carone et

al., 1985). Many of the metabolites identified by NMR that were found to be significantly increased fell-into compound classes such as of amino acids and carbohydrates, thereby corroborating previous studies (see Tables 8 and 10). Furthermore, it has been proposed that the toxic mechanism of D-serine results from oxidative stress induced by the peroxisomal oxidative deamination of D-serine by the enzyme D-amino acid oxidase to produce α-keto acids, ammonia and hydrogen peroxide (Orozco-Ibara et al., 2007). Metabolites identified as α-keto acids by NMR were also found to be significantly elevated and support, in part, the proposed oxidative stress model of D-serine nephrotoxicity.

Metabolite profiles of rat urine obtained from animals exposed to PUR were identified using NMR and LC/MS that were somewhat similar, but differences were noted. Regulated metabolites that were identified by both NMR and LC/MS included 1-methylnicotinamide, hippurate, citrate and trigonelline. Again, NMR analysis resulted in a greater numbers of metabolites being identified (see Table 13). Furthermore, some of the urinary metabolites identified by NMR and LC/MS (i.e. citrate, glucose and acetate) have been previously reported in the literature as being significantly altered following PUR exposure (Shockcor and Holmes, 2002). Further analysis will be required to confirm and validate metabolite profiles identified in the current study resulting from PUR exposure.

Identifying, cataloging, and biologically interpreting all variable metabolites that may be differentially regulated in response to toxic chemical exposure will require a great deal of effort. However, these preliminary results support the plausibility of the current strategies for development of an "Expert Software System" for analysis of biomarkers as an early indicator of potential target-organ toxicity. By complementing NMR measurements with more sensitive LC-MS profiles, it will be possible to greatly extend the scope of urinary metabolic profiling. However, this is contingent on the establishment of an LC-MS metabolite library and metabolite identification procedure.

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